New Immune Effector Pathways

in Psoriasis

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To my parents

and

my sister

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Summary

Psoriasis is a chronic relapsing inflammatory skin disease affecting 2-3% of the population worldwide. It has a strong genetic component and is present in all racial groups. Psoriasis is the result of dysregulated interactions between the skin and the immune system mediated by T cells, dendritic cells and cytokines. The most important clinical and histological features are epidermal hyperplasia, increased dilatation and growth of blood vessels together with abnormal T cell proliferation and migration into the epidermis. While psoriasis aetiology remains unknown and no cure is currently available, considerable progress in the understanding of the genetics and immunopathology has been achieved in the last years. T cells together with their secreted cytokines and chemokines are currently believed to be the primary cause of the disease. It is likely that the cellular composition of the inflammatory infiltrate within the psoriatic plaques, as well as keratinocyte hyperproliferation, is directed by cytokines. There is a complex cytokine network in psoriatic lesions consisting of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and other type I cytokines including interleukin (IL)-12 and the recently discovered IL-23 and IL-17.

Accumulating evidences point to a potential role of the two new identified cytokines, IL-23 and IL-17, in the development of inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and potentially psoriasis. The possible role of IL-23 in the pathogenesis of psoriasis is supported by the evidence that monoclonal antibodies targeting the p40 subunit of IL-23 have shown marked clinical efficacy in psoriasis patients. Moreover a single nucleotide polymorphism in the IL-23 receptor (IL-23R) gene has been associated with psoriasis. IL-17 is produced by a specific subset of T helper (Th) cells, called Th-17, and it has been shown to be involved in different animal models of autoimmunity. The first part of this study gives insights into the role of IL-23 and IL-17 during psoriasis pathogenesis and investigates their potential as targets for therapy. By analysing the blood of psoriatic patients, an increased expression of IL-23R on helper T cells and dendritic cells when compared to healthy controls was found. Furthermore high expression of IL-23R was seen on both epidermal and dermal T cells as well as epidermal and dermal dendritic cells of psoriatic patients. Significantly increased

levels of IL-17 were secreted by T cells present in the blood as well as skin of psoriatic patients compared to T cells isolated from healthy donors. Interestingly, in lesional psoriatic skin, in addition to IFN-γ producing Th1 cells and Th-17 cells, a consistent subpopulation secreting both cytokines was identified. By using a xenotransplantation mouse model with spontaneous development of psoriasis, the *in vivo* relevance of IL-23 and IL-17 in psoriasis was then analysed. Uninvolved human skin from patients with psoriasis was engrafted onto AGR mice, which are deficient in type I and type II interferon as well in the recombinase activating gene 2 (Rag2). While blocking antibodies for IL-23 inhibited psoriasis development, injection of anti-IL17 monoclonal antibodies did not have significant effects. Remarkably, the inhibition mediated by anti-IL-23 was as potent as using anti-TNF-α antibody, a "gold standard" in anti-psoriatic treatment. Taken together these results demonstrated a functional and potentially therapeutic role of IL-23 in psoriasis as well as dysregulated production of the effector cytokine IL-17.

As integrin signalling plays a critical role in many aspects of normal growth, differentiation, and injury response the second part of this work focused on the functional expression of two major collagen-binding receptors, integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Firstly, the relevance of $\alpha_1\beta_1$, the major collagen IV-binding receptor, for expansion of epidermal T cells and psoriasis manifestation was investigated. In active psoriasis lesions, $\alpha_1\beta_1$ integrin was exclusively expressed by infiltrating epidermal but not dermal CD8+ and CD4+ T cells. Interaction of freshly isolated T cells with collagen IV was shown to up-regulate the expression of $\alpha_1\beta_1$, demonstrating the positive-feedback induced by this collagen on $\alpha_1\beta_1$ expression. $\alpha_1\beta_1$ positive T cells were shown to be effector memory Th1 cells highly expressing the E-cadherin receptor $\alpha_E\beta_7$ important for T cell retention into the epidermis. Blockade of $\alpha_1\beta_1$ inhibited migration of T cells into the epidermis in the AGR mouse model and completely prevented psoriasis onset. Secondly, the important role of collagen I receptor, integrin $\alpha_2\beta_1$, during psoriasis development was analysed. Attachment of keratinocytes to the basement membrane is mediated by integrin receptors, the most abundant of which is $\alpha_2\beta_1$. Gaps or areas with reduced staining for collagen IV and laminin can be noted at the epidermo-dermal interface in psoriasis, exposing dermal collagen I to psoriatic keratinocytes expressing integrin $\alpha_2\beta_1$. The hypothesis of this project was that collagen I engagement through $\alpha_2\beta_1$ induced keratinocyte

hyperproliferation in psoriasis. *In vitro* studies showed that keratinocyte hyperproliferation on collagen I coated plates was significantly reduced by blocking $\alpha_2\beta_1$, indicating that the possible loss of basement membrane integrity in psoriasis exposed epidermal cells to collagen I and induced keratinocyte hyperproliferation in an $\alpha_2\beta_1$ dependent manner. $\alpha_2\beta_1$-collagen I interactions were shown to be also the cause of dermal endothelial cells (DMECs) hyperproliferation, as anti-α_2 monoclonal antibodies markedly reduced the proliferation rate of DMECs cultivated on collagen I-coated wells. Additionally, α_2-blockade efficiently inhibited DMECs migration and tube formation, thereby demonstrating the functional role of this integrin during angiogenesis. Finally, *in vivo* experiments using the AGR mouse model showed that blockade of $\alpha_2\beta_1$ significantly inhibited the development of psoriasis. These results demonstrated the beneficial role of blocking $\alpha_2\beta_1$ function in psoriasis and presented a T-cell independent mechanism for inhibiting the development of the disease.

In conclusion, this study gives new insights into the pathogenesis of psoriasis trying to elucidate the complex relationship between the skin and the immune system. In particular, this work demonstrated the importance of cytokines, T cells and integrin interactions during psoriasis onset and might provide the basis for new strategies in psoriasis treatment.

Zusammenfassung

Psoriasis, zu Deutsch Schuppenflechte, ist eine chronisch wiederkehrende, entzündliche Hauterkrankung, von welcher 2-3% der westlichen Bevölkerung betroffen sind. Genetische Faktoren haben einen starken Einfluß auf die Krankheitsbildung, unabhängig von der ethnischen Zugehörigkeit der Betroffenen. Psoriasis ist das Resultat einer Fehlregulation bei der Interaktion zwischen der Haut und dem Immunsystem. T-Lymphozyten, Keratinozyten, dendritische Zellen und verschiedene Zytokine sind an der Pathogenese der Psoriasis beteiligt. Die wichtigsten klinischen und histologischen Eigenschaften der Psoriasis sind eine epidermale Hyperproloferation, Dilatation und Wachstum von Blutgefässen im Zusammenspiel mit erhöhtem T-Zell-Wachstum und T-Zell-Migration in die Epidermis. In den letzten Jahren haben beachtliche Forschungsergebnisse neue und umfassendere Einblicke in die Genetik und in die immunologischen Vorgänge der Psoriasis möglich gemacht. Dennoch bleibt die Ätiologie der Psoriasis immer noch unbekannt, und eine Therapie im Sinne einer Heilung ist zur Zeit nicht verfügbar.

Die heutige Datenlage weist darauf hin, dass T-Lymphozyten und die von diesen produzierten Zytokinen und Chemokine die Krankheit auslösen. Es ist wahrscheinlich, dass sowohl das entzündliche Infiltrat innerhalb der psoriatischen Läsion als auch die Hyperproliferation der Keratinocyten durch Zytokine stimmuliert werden. In einer psoriatischen Läsion besteht ein komplexes Zytokinnetzwerk, welches sich unter anderen aus Interferon (IFN-γ), tumor necrosis factor-α, anderen Typ I Zytokinen wie Interleukin (IL)-12.

In den letzten Jahren deutet eine immer größer werdende Zahl an ForschungsePunktmutationen innerhalb des IL-23-Rezeptor (IL-23R)-Gens mit der Entwicklung der Psoriasis in Zusammenhang stehen. Ausserdem fördert IL-23 die Expansion einer Subpopulation von T-Zellen, die IL-17 produziert, den so genannten Th17 Zellen. Eine erhöhte Expression von IL-17 konnte in den letzten Jahren auf Grund von Tierversuchen mit verschiedenen Autoimmunerkrankungen assoziiert werden.

Der erste Teil dieser Arbeit gibt Einblicke in die Rolle von IL-23 und IL-17 bei der Pathogenese der Psoriasis und beschreibt des weiteren, das Potential dieser Zytokine als mögliche Zielproteine bei therapeutischen Ansätzen. Bei Analysen des Bluts von psoriatischen Patienten wurde, im Vergleich zu gesunden Probanden, eine erhörte Expression von IL-23R auf T-Zellen und dendritischen Zellen nachgewiesen. In der psoriatischen Haut zeigen sowohl epidermale und dermale T-Zellen, als auch epidermale und dermale dendritische Zellen eine erhöhte Expression von IL-23R. Im Blut und in der Haut von psoriatischen Patienten wurde des weiteren auch eine Überexpression von IL-17 durch T-Zellen beschrieben. Interessanterweise wurde in der psoriatischen Haut neben den IFN-γ produzierenden T-Zellen und den Th17 Zellen auch eine beträchtlich grosse Subpopulation von T-Zellen nachgewiesen, die beide Zytokinen ausschütteten. Die *in vivo* Rolle von IL-23 und IL-17 während der Entstehung der Psoriasis wurde in einem Mausmodell für Psoriasis (AGR Maus) analysiert. Die AGR Mäuse besitzen zusätzlich zu den fehlenden T- und B-Lymphozyten keine Typ-I- und Typ-II-Interferon-Rezeptoren und somit keine funktionellen NK(Natural Killer)-Zellen. Diese AGR Mäuse sind optimale Empfänger für die Transplantationsexperimente, bei denen nicht-involvierte Haut eines Psoriasis-Patienten auf den Rücken der Mäuse transplantiert wird. Innerhalb von 5 Wochen nach der Transplantation entwickelt sich die nicht involvierte Haut zu einem psoriatischen Phänotyp, der alle charakteristischen histologischen Merkmale der Psoriasis zeigt. Die Blockierung von IL-23 durch neutralisierende Antikörper verhindert die Entstehung des psoriatischen Phänotyps in den Transplantaten, wogegen die Entwicklung einer Psoriasis durch die Hemmung von IL-17 mit monoklonalen anti-IL-17 Antikörpern nicht inhibiert werden konnte. Diese Ergebnisse zeigen klar das Potential, welches eine Blockierung von IL-23 als mögliche Therapie für Psoriasis Patienten hat und beweist die dyrgebnissen darauf hin, dass die kürzlich beschriebenen Zytokinen, IL-23 und IL-17, eine wichtige Rolle in der Entwicklung von Autoimmunerkrankungen, wie Rheumatoide, Arthritis, multiple Sklerose und Psoriasis spielen. Ein neuer Therapieansatz, welcher die Funktion der p40 Untereinheit der Zytokine IL-12/IL-23 blockiert, hat beeindruckende klinische Resultate in der Behandlung der Psoriasis gezeigt und weist gleichzeitig auf eine Schlüsselrolle dieser Zytokine bei dem Krankheitsverlauf hin. Es konnte gezeigt werden, dass zwei verschiedene sregulierte Produktion des Effektor-Zytokins IL-17.

Da Integrine bei normaler Proliferation, Differenzierung und in der Wundheilung eine zentrale Rolle spielen, wurde in dem zweiten Teil dieser Arbeit die Expression der Kollagensrezeptoren $\alpha_1\beta_1$ und $\alpha_2\beta_1$ untersucht. Zuerst wurde die Bedeutung von $\alpha_1\beta_1$, dem wichtigsten Kollagen IV Rezeptor, während der Expansion von epidermaler T-Zellen und der Entstehung der Psoriasis analysiert. In der betroffenen psoriatischen Haut wurde $\alpha_1\beta_1$ Integrin nur auf eingewanderten, epidermalen und nicht auf dermalen CD8+ und CD4+ T-Zellen nachgewiesen. Die Interaktion von frisch isolierten T Zellen mit Kollagen IV führte zu einer gesteigerten Regulation von $\alpha_1\beta_1$. Dieses Experiment demonstriert die positive Rückkopplung, induziert durch Kollagen IV, auf die Expression von $\alpha_1\beta_1$. Es wurde gezeigt, dass $\alpha_1\beta_1$ T-Zellen zu der Klasse der Effektor-Gedächnisszellen gehören und Th1 Zellen sind. Diese $\alpha_1\beta_1$ T-Zellen exprimieren den E-Cadherin Rezeptor $\alpha_E\beta_7$, welcher für die Anhaftung von T-Zellen an die Epidermis wichtig ist. Die Hemmung von $\alpha_1\beta_1$ in dem AGR Maus Modell blockierte die Einwanderung von T-Zellen in die Epidermis und inhibierte die Entstehung einer Psoriasis. Im zweiten Teil dieses Projektes wurde die Rolle des Kollagen I Rezeptor (Integrin $\alpha_2\beta_1$), während der Entwicklung einer psoriatischen Läsion analysiert. Keratinozyten binden an die Basalmembran durch Integrin-Rezeptoren. Unter diesen ist der Integrin $\alpha_2\beta_1$ der am stärksten exprimierte Rezeptor. Immunfärbungen zeigen Lücken oder sogar ganze Areale mit verminderter Färbung für Kollagen I und Laminin, besonders an der epidermo-dermalen Grenze in psoriatischer Haut. Dadurch wird dermales Kollagen I an die Keratinocyten präsentiert, welche Integrin $\alpha_2\beta_1$ exprimieren. Die Hypothese welche diesem Projekt zu Grunde liegt, ist das die Interaktion von Integrin $\alpha_2\beta_1$ mit Kollagen I zu einer Hyperproliferation der Keratinozyten führt. *In vitro* Experimente zeigten, dass die Blockierung dieses Integrins die Hyperproliferation der Keratinocyten und die Angiogenese systemisch reduziert. Um die *in vivo* Relevanz zu untersuchen, wurden die Experimente in dem AGR Mausmodell durchgeführt. Mäuse, die mit anti-α_2-Antikörper injiziert wurden, zeigten keine Hyperproliferation der Keratinocyten und die Entstehung ein psoriatischen Phänotyp wurde vollständig inhibiert. Da während der Entwicklung der Psoriasis keine Expression von $\alpha_2\beta_1$ auf T-Zellen nachgewiesen werden konnte, scheint die Blockierung der Psoriasis durch anti-α_2-Antikörper ein T-Zellen unabhängiger Mechanismus zugrunde zu liegen.

Zusammenfassend gibt diese Arbeit einen Einblick in die Pathogenese der Psoriasis und versucht den komplexen Zusammenhang zwischen der Haut und dem Immunsystem zu erläutern.

Insbesondere zeigt diese Arbeit wie wichtig die Interaktion zwischen Zytokinen, T-Zellen und Integrinen für die Pathogenese der Krankheit sind und liefert somit die Basis für neue, therapeutische Strategien zur Bekämpfung der Psoriasis.

Abbreviations

Ab	Antiobody
AGR	mice deficient in type I (A) and type II (G) IFN receptors, in addition to be RAG-2$^{-/-}$
APC	Antigen Presenting Cells
APC (Staining)	Allophycocyanin
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CIA	Collagen Induced Arthritis
CNS	Central Nervous System
DC	Dendritic cell
DMECs	Dermal Microvascular Endothelial Cells
EAE	Experimental Autoimmune Encephalomyelitis
ECs	Endotjelial Cells
ECM	Extracellular Matrix
FACS	Fluorescent Associated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Growth Factor
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
IL-23R	IL-23 receptor
LC	Langerhans cells
KC	Keratinocytes
mAb	Monoclonal antibody
MACS	Magnetic Activated Cell Sorting
MHC	Major Histocompatibility Complex
NK	Natural Killer
NN	Skin of healthy volunteers
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phodphate Buffered Saline
PE	Phycoerythrin
PHA	Phytohemagglutinin

PI	Propidium Iodide
PMA	Phorbol-12-Myristate-13-Acetate
PN	Non involved Psoriatic skin
PP	Invovlved Psoriatik skin
RAG-2	Recombinase Activating Gene 2
RT-PCR	Real Time Polymerase Chain Reaction
SDF-1α	Stromal cell-Derived Factor 1α
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen

Chapter 1

General introduction

1. General introduction

1.1. Introduction to skin biology

The skin is a complex barrier organ, which separates internal structures from the outside environment. Its essential functions include protection against external environmental insult, maintenance of fluid homeostasis and thermoregulation. The skin is composed of three structurally and functionally distinct layers, the epidermis, the dermis and the subcutis (Fritsch 2004) (Figure 1.1).

The epidermis forms the outer most layer and it can be further subdivided into five recognisable strata or layers. From the inner to the outer they are respectively the stratum basale, spinosum, luicidum, granulosum, and corneum. Each of these layers is primarily composed of keratinocytes at various differentiation stages that are derived from the basal layer. Following cell division some keratinocytes remain in the basal layer as stem cells while others move up towards the surface. During this migration the keratinocytes gradually flatten, lose their nuclei and most other structures to finally leave only the keratin cytoskeleton to make up the stratum corneum. This keratinisation process takes approximately four weeks after cell division at the basal layer (Eckert and Rorke 1989). Keratinocytes not only compose the scaffold and structure of the epidermis, but they also take active part in its inflammatory and immunological reactions. During normal homeostasis keratinocytes secrete few factors, but in response to wounding or infection they release a broad spectrum of proinflammatory cytokines such as IL-1, IL-6, TNFα, GM-CSF, IL-8, MCP-1 and TGF-β thereby initiating or propagating inflammatory and immune reactions (Fritsch 2004). In addition to keratinocytes, the epidermis is also populated by smaller numbers of other cells, such as melanocytes and Langerhans cells. Melanocytes sit in the basal layer and produce melanin primarily in response to ultraviolet radiation. Using large numbers of dendrite-like processes melanocytes distribute melanin to the surrounding keratinocytes, protecting their nuclei from further UV-damage. Melanin is principally responsible for pigmentation of the skin. Langerhans cells are antigen presenting cells which contribute to protect the skin

from infection, by capturing and delivering various antigens to lymphoid tissues where they can activate naïve T lymphocytes (Janeway 2001). While the epidermis contains no blood vessels, its cells are supported by the diffusion of nutrients from dermal capillaries.

A basement membrane separates the epidermis from the dermis and provides a structural support for melanocytes and proliferating keratinocytes. This layer is composed of the lamina lucida and lamina densa. The lamina lucida is adjacent to the epidermis and consists of laminin and collagen type IV; while collagen type VII is the main constituent of the lamina densa.

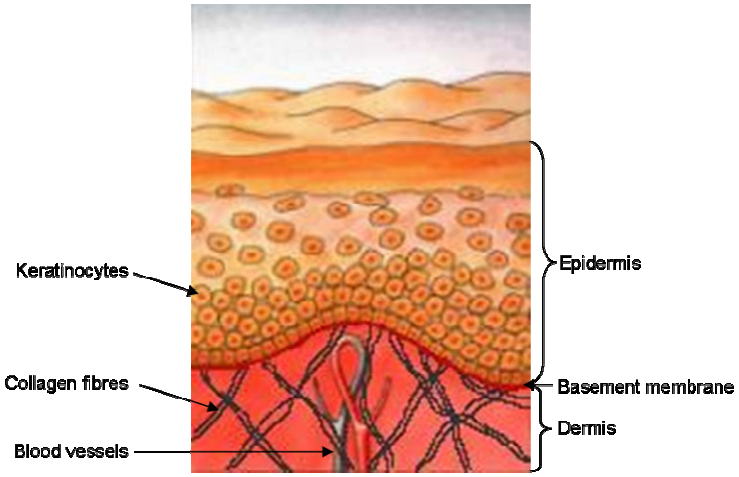

Figure 1.1 Structure of the skin Skin is composed of the epidermis and dermis, which are separated by the basement membrane. As indicated, keratinocytes, collagen fibres and blood vessels are present in these two layers.

Beneath the basement membrane lies the dermis which principally consists of a dense layer of fibrous connective tissue that gives the skin its flexibility and strength. The dermis can be subdivided into the stratum papillare and the deeper stratum reticulare, which show a continuous transition to subcutis. The dermis is rich in a network of nerves and vasculature. Embedded within the dermis are several different cells types, including fibroblasts, mast cells, various antigen presenting cells and lymphocytes. Fibroblasts are responsible for secretion of the collagen and elastin fibers that constitute the extracellular matrix of connective tissue. Mast cells are found concentrated around blood and lymphatic vessels and together with antigen presenting cells they represent one of the first lines of defense against pathogens.

The subcutis, also called hypodermis, is the deepest layer of the skin. Attached to the dermis by collagen and elastin fibres it divides the dermis from muscle, bone and

other tissues. This layer is mainly composed of adipocytes, a type of cell specialised for the accumulation and storage of fat.

1.2. The skin immune system

As a protective interface between the body and the external world, the skin functions not only as a physical barrier, but it is also an active immune organ. Cutaneous immune responses involve the coordinate action of immune cells distributed through all the layers of the skin. In 1978, Streilein was among the first to propose a branch of the immune system specialized to provide skin immunity, that he named skin associated-lymphoid tissues (SALT) (Streilein 1983). Skin associated-lymphoid tissues include epidermal and dermal cells that work together to provide immune protection against microbial, chemical and physical insults.

1.2.1. General concepts in immunology

1.2.1.1. Human immune system

The immune system consists of two functional arms classified as innate and adaptive immunity. *Innate immunity* is a first line body defence and is characterised by a rapid but unspecific response and by the lack of immunological memory. The components of the innate immune response are constitutively present and always ready to be mobilised upon infection. To protect the host upon infection the innate immune system uses both physical barriers, such as the skin and the mucosal epithelium, and rapid cellular responses enhanced by monocytes, natural killer cells, antigen presenting cells and granulocytes. These cells express pattern-recognition receptors that recognise specific pathogens components. An important subset of this receptors belong to the *Toll-like receptor* (TLR) family, which bind pathogen associated molecular pattern (PAMP) molecules such as peptide molecular structures from bacteria, single- and double-stranded RNA from viruses, and yeast mannans (Gay, Gangloff et al. 2006). TLRs are transmembrane glycoproteins expressed on the cell surface and endosomally and are capable of initiating innate immune responses and influencing subsequent adaptive immune responses. Because *adaptive immunity* is tailored to the antigen (antigen- specific) it takes more

time to be activated, but is highly specific and has a memory, so that subsequent exposure to the same antigen leads to a more effective and rapid response. Key players during this response are antigen-presenting cells and T lymphocytes (Schwarz 2003).

1.2.1.2. Antigen presentation

The function of the *major histocompatibility complex (MHC)* is to bind peptides in an intracellular location and display them on the cell surface for the recognition by the appropriate T cell. The MHC genes are the most polymorphic genes known, as there are multiple variants of each gene within the population. Moreover, every individual has a set of MHC molecules with different ranges of peptide-binding specificities.

There are two types of peptide-MHC complexes: class I molecules bind peptides from proteins degraded in the cytosol, such as viral or tumour proteins, and are only recognised by CD8+ T cells. While class II molecules process exogenous antigens taken up by endocytosis and are recognised by CD4+ T cells.

Class I molecules are found on almost all nucleated cell of the body, while class II proteins are mainly expressed by antigen presenting cells.

Antigen presenting cells (APC) are highly specialised cells that serve two major functions during adaptive immunity. First they capture and process antigens on their cell surface together with MHC molecules required for lymphocytes activation and second they express receptors (CD80, CD86 and CD40) required for the proliferation and differentiation of lymphocytes. APC include dendritic cells, macrophages and B cells.

Dendritic cells (DCs) are the most important professional APC for the induction of primary immune responses and are the key link between the innate and adaptive immune system.

Dendritic cells start out as immature dendritic cells (iDCs) which are characterized by high endocytic activity and low T-cell activation potential. DCs utilize several pathways to capture antigen including pinocytosis, receptor mediated endocytosis

and phagocytosis. Captured antigens are then processed in distinct intracellular compartments and loaded onto MHC molecules. By the time iDCs process the antigen they leave the skin, enter lymphatic vessels and migrate to the nearest lymph node where they arrive as mature DCs. This maturation process enhances antigen processing and up-regulates the expression of MHC and surface molecules, known as co-stimulatory molecules (CD80, CD86 and CD40) which are fundamental for T cell activation.

There are two major subsets of dendritic cells in humans: plasmacytoid DCs (pDCs) and myeloid (mDCs), including dermal DCs and Langerhans cells (LCs) (Banchereau, Pascual et al. 2004). In accordance to their different TLR expression pattern these two subsets of DCs respond to different pathogen-associated molecular patterns.

The only TLR members expressed on pDCs are TLR-7 and-9, which are intracellular receptors specialized in the recognition of viral nucleic acids. In contrast, mDCs express a broader profile of TLR molecules, including the surface expressed TLRs (TLR-1, TLR-2, TLR-4 and TLR-6) that are mainly involved in the recognition of bacterial components and the intracellular TLR-3 and TLR-8, which bind double-stranded RNA from viruses (Seya, Akazawa et al. 2006).

PDC have low capacity to activate naïve T cells and represent key effectors in the innate antiviral immunity. They are characterized by their capacity to secrete large amounts of IFN-α in response to viruses and have also be found to play a role during autoimmune disease development (Nestle, Conrad et al. 2005). During homeostasis pDCs are found at low frequencies in the blood and secondary lymphoid organs but upon viral infection they are recruited to the site of inflammation. Finally, virally triggered pDCs differentiate into mature DCs able to induce the differentiation of CD4+T cells able to eliminate infected cells. By contrast, mDCs have a high capacity to activate naïve T cells and are mainly activated by microbial signals such as lipopolysaccharides or CpG bacterial DNA motives. They are usually located at the interface between outer world and the body such as the skin, the respiratory tract and the gastrointestinal tract. After activation mDCs produce IL-12 which is a dominant cytokine involved in the development of cytotoxic T cells.

Macrophages provide a first line of defence against infection. They can also be activated to express co-stimulatory and MHC class-II molecules. This enables them to work as APC, even if they are less potent in activating T cells. In some situation, *B cells* can also function as APC. They contribute to adaptive immunity by presenting peptides from antigens that they have ingested and by secreting antibodies.

1.2.1.3. T cells and self-tolerance

T cells are the main effectors of the adaptive immune response and they only recognise antigen-peptides presented in the groove of host MHC molecules. This recognition occurs through the T cell receptor (TCR), a heterodimeric protein composed of an α and β chain, or less frequently of a γ and δ chain. Stably associated with the TCR on cell surface is the CD3 complex, which signals to the interior of the cell upon antigen binding. Among the α/β T cells are two important subpopulations: CD4+ T- helper cells (Th) and CD8+ T cytotoxic (Tc) cells. These two subpopulations differ fundamentally in how they recognise antigen and for the different types of regulatory and effector functions. The differentiation of T cells in CD4 versus CD8 occurs during their development in the thymus (Janeway 2001). During this stage T cells are also "educated" to tolerate the host molecular structures (self) and therefore react only when foreign peptides are bound to MHC molecules.

T cells originate from the bone marrow and then migrate to the thymus where they are called thymocytes. Thymocytes do not express specific T cell surface markers (TCR, CD3 and CD4/CD8) and are called double negative cells (because they are CD4-CD8-). During T cell development a first critical step is the selection for a functional T cell receptor, which occurs when T cell precursors pass through the thymic cortical region. Cells that successfully pass this stage become double positive cells (CD4+CD8+). Double positive thymocytes are then subjected to the other two selection processes, called positive and negative selection. During positive selection only progenitors that have low affinity for self MHC are selected for proliferation, the others undergo programmed cell death (apoptosis). This stage assures also that thymocytes which are MHC-I restricted are positively selected towards CD8 ligands whereas the MHC-II restricted cells are positively selected to the CD4 lineage

(Sebzda, Mariathasan et al. 1999). Finally, negative selection leads to the deletion of thymocytes, whose T cell receptors have too high affinity for self antigens.

1.2.1.4. T cell activation

After their export from the thymus, T cells, called naïve T cells, enter the bloodstream and start a constant recirculation through secondary lymphoid organs and the blood, until they encounter a foreign antigen.

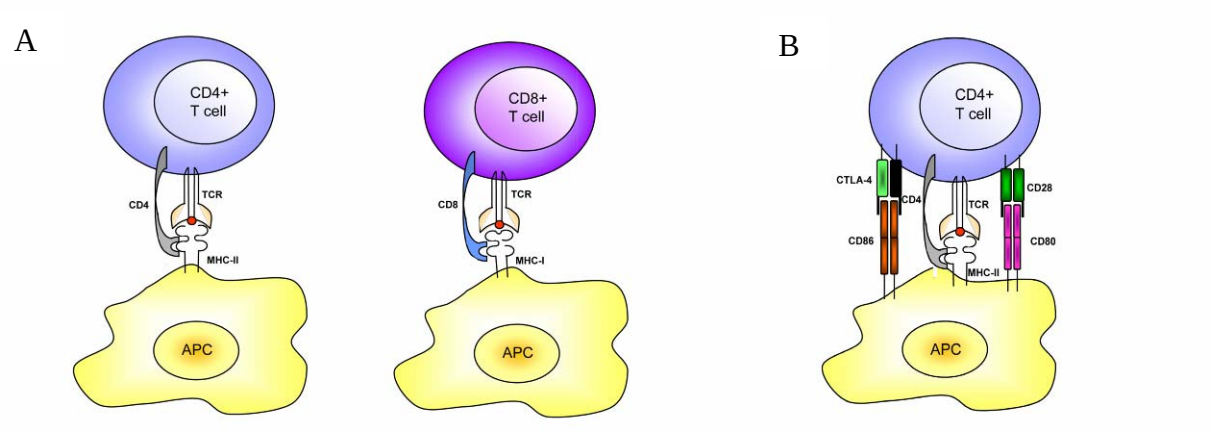

Figure 1.2 Signals for T cell activation. (A) First interaction: molecular association of CD4+ T cells with MHC-II and CD8+ with MHC-I. (B) Costimulatory signals necessary to continue the immune response

In order to be activated naïve T cells require two signals (Figure 1.2). The first signal arises from the interaction of their TCR with the specific MHC/antigen complex expressed on APCs. During this event CD3 molecules signal to the interior of the cells that recognition has occurred. However this interaction alone fails to induce complete T cell activation. An additional second signal, derived by co-stimulatory molecules expressed on APC, is needed. The main co-receptors for T cell activation are CD80, CD86 and CD40 that respectively bind CD28, CTLA-4 and CD40 ligand on T cells. Antigen binding to the TCR in the absence of co-stimulation does not activate the cell but rather leads to cell death or to a state called anergy, by which T cells become functionally inert (Parkin and Cohen 2001). If both stimulatory signals are present, T cells start to proliferate and secrete interleukin-2 (IL-2), a cytokine that promotes T-cell growth in an autocrine fashion by binding to its receptor IL-2R (Janeway 2001). During this proliferation some of the T cells will differentiate into

effector T cells while another fraction will form memory T cells that will survive in an inactive state until they re-encounter the same antigen that will reactivate them.

1.2.1.5. Effector T cells

There are two major types of effector T cells, T helper cells (Th) or T cytotoxic cells (Tc). Th cells bear CD4 molecules and are the main cells of the adaptive immune response. Tc cells are CD8+ cells able to destroy virally infected cells, tumour cells and are also implicated in tumour rejection.

Th cells can further be divided in Th1 or Th2 cells, according to the type of cytokines they secrete. Whether a CD4 T cell will differentiate into a Th1 or Th2 is determined by the nature of the antigen recognised, the co-stimulators used to drive the response and the cytokine environment (Janeway 2001). Th1 cells typically secret IFN-γ, TNF-α and IL-2. They are mostly generated upon infection with intracellular pathogens such as viruses or mycobacteria. These pathogens are recognised by TLR on the surface of macrophages and DC, which consequently produce IL-12. Binding of IL-12 to its receptor on CD4+ T cells activates the transcription factor T-bet and STAT-4, and these promote the differentiation in Th1 cells.

Th2 cells, instead, secrete IL-4, -5 and -13 and their development depends on the IL-4 cytokine. Binding of IL-4 to its receptor on CD4+ cells induces the transcription of STAT-6 and GATA-3 which are essential for Th2 differentiation. Th2 cells mainly mediate anti-parasitic and allergic responses. It is important to note that cytokines produced by one subtype function as negative regulator of the other subtype's development and function (Paul 1999).

Tc cells recognise the antigen in association with MHC-I molecules and are directly cytotoxic to cells infected with viruses (or other pathogens), or are otherwise dysfunctional. There are two mechanisms by which activated Tc cells kill target cells. One method involves release of secretory granules containing perforin and granzyme, two proteins capable of inducing the lysis of target cells. The second mechanism is Fas-mediated apoptosis. Tc cells express on their surfaces a death activated protein, called Fas Ligand, which bind to its receptor, called Fas, present on almost all target cells. When Tc cells recognise a target cell, they overexpress Fas

ligands, which bind with the Fas receptor present on the target cell leading to its death.

In the last years increasing interest has been focused on regulatory T cells (*Tregs*), previously called “suppressor T cells”. Tregs participate in suppressing autoimmune reactivity against self structures, in controlling microbial infection, allergy and facilitating transplantation tolerance (Sakaguchi 2006).

Several distinct subsets of Treg cells have been described based on distinct expression patterns of surface markers and cytokines. The best characterized Tregs in humans and mice are the so called naturally occurring Tregs (nTregs) a subset of CD4+ T cells constitutively expressing CD25, the IL-2 receptor α chain. nTregs can also be identified by their expression of FoxP3 that encodes the forkhead box P3 transcripiton factor, a key gene in their development and function. Typically high levels of CTLA-4 (cytotoxic T lymphocyte antigen 4), known as a negative regulator of T cell activation, and GITR (glucocorticoid-induced TNF receptor) are also expressed on this cell population (Tai, Cowan et al. 2005).

One well accepted function of CD4+CD25+ natural Tregs is the maintenance of self-tolerance suppressing the activation and function of autoreactive T cells, thereby preventing autoimmune diseases. After TCR-mediated stimulation, nTregs cells suppress the activation and proliferation of other CD4+ and CD8+ cells in an antigen-non specific manner, via mechanisms that require cell-to-cell contact. In mice, depletion of CD4+CD25+ T cells by neonatal thymectomy leads to spontaneous development of organ-specific autoimmune disease, condition that can be reversed by adoptive transfer of Treg (Asano, Toda et al. 1996).

Treg were originally considered to derive only from thymic precursors, to be exported to the periphery, and to represent less than 10% of CD4+T. Further studies (Wahl, Swisher et al. 2004) showed that Treg can be also expanded and induced in the periphery from CD4+CD25- naive T cells. This conversion is driven by TGF-β which is a physiologic inducer of Foxp3. When co-activated with TCR and TGF-β, CD4+CD25- cells begin to express the transcription factor Foxp3, which is the master regulator for their transition into CD4+CD25+ Treg cells. This conversion may be happen in a site of autoimmune reactivity in order to increase locally the number of Tregs cells.

Recently it has become evident that TGF-β is also involved in the generation of a recently discovered cell population called *Th-17* (Bettelli, Carrier et al. 2006; Veldhoen, Hocking et al. 2006). Th-17 cells are well accepted to be involved in the pathogenesis of certain mice autoimmune diseases such as rheumatoid arthritis, experimental autoimmune enchemalomyelitis (EAE) and allergen-specific responses (Nakae, Komiyama et al. 2002; Cua, Sherlock et al. 2003; Langrish, Chen et al. 2005). IL-17 is believed to contribute to the pathogenesis of these diseases by acting as a potent proinflammatory mediator. The molecular mechanism inducing the development of Th-17 cells is still unclear. Recently it has been shown that both TGF-β and IL-6 are required for the full differentiation of Th-17 cells from CD4+ T cells *in vitro*. These recent discoveries suggest that nTreg and Th-17 cells have close affinity and that both T cells subsets may differentiate from the same precursor depending on the type of cytokines present in the local environment (Cua and Kastelein 2006).

1.2.1.6. Memory T cells

Memory T cells are faster and more effective responders to infection then naïve T cells. They also respond to a lower antigen dose and are less dependent on co-stimulatory signals. In the peripheral blood, two types of memory T cells have been described based on the homing molecules they express. Central memory T cells (T_{CM}) express L-selectin (CD62L) and the chemokine receptor CCR7 for efficient traffic through secondary lymphoid tissues. In contrast, effector memory T cells (T_{EM}) lack these migration receptors and are therefore excluded from these organs and are found in non-lymphoid tissues (such as the gut, lung and liver) (Lanzavecchia and Sallusto 2005; Lefrancois and Marzo 2006). Another important property of memory T cells is that after re-exposure to antigen, they specifically home to the site where the initial antigen exposure had taken place. This selective migration is due by the differential expression of adhesion molecules. In this way memory T cells that home to the gut express the integrin $\alpha_4\beta_7$ which bind to the E-cadherin expressed on epithelial cells. Instead, cells that home to the skin express the cutaneous lymphocyte antigen (CLA), whose ligand is the skin E-selectin.

1.2.2. Normal immune surveillance in the skin

The cutaneous immune surveillance arises from the interaction of innate and adaptive immune systems (Kupper and Fuhlbrigge 2004).

Langerhans cells (LCs) are the APCs found in the epidermis. They originate from the bone marrow and migrate into the skin, where they constitute the 2-4% of epidermal cells (Parkin and Cohen 2001). LCs are mainly localized at the suprabasal region of the epidermis and are characterized by their dendritic shape and by Birbeck granules, which are rod- shaped organelles.

In non-inflamed epidermis LCs are the only cells which express MHC-II molecules constitutively. LCs play a double function in the skin immune system as they can process antigens by picking up the antigen and migrate to the draining lymph node where they activate native T cells. But they can also activate memory/effector T cells specific for previously encountered antigens directly in the epidermis.

Circulating cutaneous lymphocytes antigen (CLA)-positive T cells represent 10-15% of all circulating memory T cells in peripheral blood and are crucial to the initiation and execution of skin immune responses (Robert and Kupper 1999). CLA+ T cells may be positive for either CD4 or CD8, and upon activation they may induce either a Th1 or a Th2 response.

E-selectin the endothelial ligand for CLA, and ICAM-1 are constitutively expressed at low levels on cutaneous endothelia and may mediate CLA+ T cells trafficking also in non inflamed skin. Those cells that encounter the specific antigen presented by local APC will be activated to proliferate and carry out their specific functions. CLA+ T cells that do not encounter their specific antigen will return to the peripheral blood through the lymphatics.

Recently it has been shown that the majority (80%) of CLA+ memory T cells reside in the skin and only a minority (20%) is recalculating. It has been calculated that in the entire non inflamed skin surface there are about 2×10^{10} T cells (1×10^{6} T cells/cm^2), nearly twice the number of T cells in the circulation (Clark, Chong et al. 2006). This observation suggests that all the components necessary for an adaptive immune response (LCs, dermal DCs and memory T cells) are already present in the skin and are sufficient to activate an efficient defence against pathogens. On the

other hand this scenario may also contribute to the development and perpetuation of inflammatory skin diseases.

After tissue damage or pathogen invasion both resident innate immune cells (LCs, dermal DCs and mast cells) and skin cells (keratinocytes and fibroblasts) are activated. Keratinocytes play an important role in cutaneous immune responses. After an injury they release different types of cytokines, including TNF-α and IL-1α which induce the up-regulation of MHC-II molecules on LCs and dermal DCs, increasing their efficiency as antigen-presenting cells. These cytokines also act on the local endothelia increasing the expression of cellular adhesion molecules (E-selectin, P–selectin, intracellular adhesion molecule 1(ICAM-1)). The expression of all these molecules enables circulating leukocytes (neutrophils, natural killer cells and effector T cells) to extravasate to the site of skin injury (Kupper and Fuhlbrigge 2004). In this way CLA+ memory T cells by binding to E selectin and other adhesion molecules are recruited to the skin in an antigen non-specific manner. Once in the skin, T cells respond to chemotactic gradients released by cells in the side of injury. If T cells encounter the cognate antigen, they will respond inducing a rapid and effective immune response.

1.2.3. Autoimmunity

Autoimmunity is the failure of the organism to recognise its own constituent parts as no-self, which results in an immune response against its own cells and tissues. This aberrant response is due to autoantibodies and/or autoreactive T cells to specific antigen within a target organ. In some autoimmune diseases the autoantigens have been already identified as in the case of rheumatoid arthritis (RA) and systemic lupus erythematousus (SLE). In the case of psoriasis several autoantigens (keratin 13, heterogeneous nuclear ribonuclearprotein-A1 and rab coupling protein isoform 3) has been proposed, but a definitive final autoantigen has not been found yet (Bowcock 2005). However an autoimmune disease becomes manifest in less 50% of genetically predispose people indicating that other factors are also needed in order to make the disease manifest. In fact environmental factors in association with defects in immunoregulatory mechanisms are the additional components that can lead to the development of an autoimmune disease in

genetically predisposed individual. Several factors are known to trigger the immunological process leading to psoriasis including streptococcal infection, trauma to the skin, drugs and alcohol consumption, smoking and stress.

1.3. Psoriasis

The name psoriasis derives from the Greek, *psora*, which means to itch. Over the centuries psoriasis was described as a variety of leprosy. In the late 18th century an English dermatologist, Robert Willan, for the first time recognised psoriasis as an independent disease. He identified two categories: *Leprosa Greacorum*, which described the condition when the skin had scale and *Psora Leprosa*, when the disease becomes eruptive. But only in 1841 Ferdinand von Herba, a Viennese dermatologist, described the clinical picture of psoriasis which is used today and ascribed it the name “psoriasis”. It is during the 20th century that psoriasis was further differentiated into specific types (Bologna JL 2003).

1.3.1. Epidemiologic and genetic features

Psoriasis affects 2-3% of the population worldwide, with prevalence varying according to race and geographic location. Higher rates of psoriasis have been reported in people of the Faroe Islands, were 2.8% of the inhabitants were found to be affected (Lomholt 1964). By contrast in Japan the prevalence is very low (0.2%) and almost absent in American Indians (Bowcock and Barker 2003).

Males and females are equally affected and based on an important epidemiology study, done by Henseler and Christophers, two types of psoriasis have been described (Langley, Krueger et al. 2005). Early onset type psoriasis (or type I) is the most common form and occurs before age 40; late onset type psoriasis (or type II) begins after the age of 40 years. Early onset type psoriasis has been reported to have more genetic susceptibility and hereditary association than late onset of the disease. The role of hereditary transmission in the pathogenesis of psoriasis is still poorly understood. Epidemiologic studies have confirmed that genetic factors are strongly involved in the pathogenesis of this disease and showed that there is a

threefold increased risk of psoriasis in monozygotic twins compared to fraternal twins (Farber and Nall 1974; Elder, Nair et al. 1994). However, as the concordance for psoriasis is never 100% among monozygotic twins these studies suggest that environmental factors also play an important role. So far at least six different susceptibility loci (designed PSORS1-PSORS6) have been identified (Nickoloff and Nestle 2004), however only two of these loci, PSORS1 and PSORS2, have been replicated in more then one study (Table I). PSORS1 is located in the MHC region on chromosome 6p21 and has been estimated to account for 30-50% of psoriasis cases (Trembath, Clough et al. 1997). For many years the HLA-Cw*0602 allele (most important gene of the PSORS1 locus) has been considered the major predisposing factor in this region. However,, due to the linkage disequilibrium in the PSORS1 region, it is difficult to ascertain that HLA-Cw6 is the relevant candidate gene. Other candidate genes in the PSORS1 region include corneodesmosin (CDSN) and α−helix coiled coil rod homolog (HCR), which are expressed in skin cells (Elder 2006). PSORS2 is located on human chromosome 17q24-q25 and different family studies have shown its involvement in psoriasis (Nair, Henseler et al. 1997; Samuelsson, Enlund et al. 1999; Helms, Cao et al. 2003). In this region two genes, called SLC9A3R1 and NAT9, may be candidate genes for psoriasis. SLC9A3R1 (solute carrier family 9, isoform 3 regulator 1) encodes a protein involved in actin cytoskeleton reorganization. It is implicated in negative selection of T cells in the thymus and may lead to the development of autoreactive T cells. NAT9 (N-acetyltransferase family member 9), is involved in the glycosylation of immunoregulatory proteins, such as MHC-I molecules and components of the immunological synapse (Nickoloff and Nestle 2004). Moreover, between the two genes SLC9A3R1 and NAT9 lies an intragenic region also of interest for psoriasis. This region contains several runx-related transcription factor (RUNX) binding sites which are involved in haematopoietic cell development, development of T cells in the thymus and gene silencing. In psoriatic patients genetic analyses of this region have identified an associated single nucleotide polymorphism (SNP) that consequently abolishes a binding site for the transcription factor RUNX1. Alteration in RUNX1 binding may lead to dysregulated self-tolerance and perturbation of T cell development or activation. Aberrant RUNX1 expression has also been correlated with other two autoimmune diseases: systemic lupus erythematosus and rheumatoid

arthritis (Tokuhiro, Yamada et al. 2003) (Alarcon-Riquelme 2004) suggesting an important role of RUNX family members in the development of autoimmunity. Psoriasis may not be classified as a classical autoimmune disease, however there are some common features between autoimmune conditions and psoriasis. For example the presence of autoreactive T cells (Nestle, Turka et al. 1994), numerous oligoclonally expanded T cells, immunosuppressing drugs such as cyclosporine are effective against psoriasis and appearance or disappearance of psoriasis after bone marrow transplantation (Snowden and Heaton 1997) (Valdimarsson, Baker et al. 1995).

Table 1.1 PSORS Loci associated with psoriasis

PSORS LOCUS	***CHROMOSOME LOCATION***	***ASSOCIATED GENE***	***STUDY POPULATION***
PSORS1	6p21	MHC class I	Many
PSORS2	17q25	SLC9A3R1 and NAT9	American American and British
PSORS3	4q	ND	-
PSORS4	1q21	Epidermal differentiation complex	Italian and American (unpublished)
PSORS5	3q21	SLC12A8	Swedish
PSORS6	19p13	ND	-

Adaptation from Bowcock et Krueger (Bowcock and Krueger 2005)

1.3.2. Clinical and histopathological aspects

Psoriasis is a chronic skin disease characterised by the presence of dry red, raised, scaly plaques, which can be several centimetres in diameter. Usually, the skin is covered with many individual lesions, separated by normal appearing skin, whereas in severe cases all the skin can be affected. The most specific histopathological changes that distinguish psoriasis from other inflammatory skin diseases are dramatic hyperplasia of the epidermis (acanthosis) with loss of the granular layer, regular elongation of the rete ridges, thickening of the cornified layer (hyperkeratosis) and incomplete keratinocyte differentiation (parakeratosis), infiltration of many different leukocytes and increased vascularity in the dermis (Bologna JL 2003).

Psoriatic lesions can be very variable in morphology, distribution and severity. Despite the classical presentation described above, psoriasis may be classified in

three main types: non-pustular psoriasis, pustular psoriasis and psoriatic arthritis. Each of these types can further be subdivided in other clinical variants (Figure 1.3) and the most common forms are outlined below.

1.3.2.1. Non-pustular psoriasis

Plaque-type psoriasis or psoriasis vulgaris is the most frequent type of psoriasis (80-90% of cases). The classical sites of involvement are elbows, knees, scalp, buttocks and the lumbar region, but all areas of the body may be affected. The lesion may initially begin as small red papules that subsequently scale as they grow larger. These plaques tend do be symmetrical with clearly defined edges.

Figure 1.3 Clinical aspects of psoriasis. A) Psoriasis Guttate. B) Psoriasis palmo plantar pustulosa. C) Plaque type Psoriasis. D) Psoriasis Erythrodermie E) Psoriasis capillitii. F) Psoriasis Arthritis. G) Nail psoriasis.

The second most common clinical form is the *guttate* phenotype which more commonly affects children or young adults and often follows a streptococcal infection. The lesions, round to oval in shape, have a very small diameter and usually spread over the trunk and the proximal limbs. This type of psoriasis has a more acute clinical course and may dissolve spontaneously. However it may eventually progress into plaque phenotype (Langley, Krueger et al. 2005).

Other less common variants are: *inverse psoriasis*, with non-scaling lesions located in groin and axillary regions and *erythrodermic psoriasis*, covering the entire body surface (Griffiths, Christophers et al. 2007).

1.3.2.2. Pustular psoriasis

Pustular psoriasis is an uncommon form of psoriasis consisting of widespread pustules on an erythematous background. Cutaneous lesions characteristic of psoriasis vulgaris may be present before, during, or after an acute pustular episode. It can affect the areas of the feet and the hands (palmo-plantar pustulosis) or be generalised and usually manifests itself as small sterile bumps or *pustules* on the surface of the skin.

1.3.2.3. Psoriatic arthritis

About 10-30% of people with psoriasis also develop psoriatic arthritis, which causes pain, stiffness and swelling in and around the joints. The onset of psoriatic arthritis generally occurs in the fourth and fifth decades of life. Males and females are affected equally. The skin disease (psoriasis) and the joint disease (arthritis) often appear separately. In fact, the skin disease precedes the arthritis in nearly 80% of patients. The arthritis may precede the psoriasis in up to 15% of patients.

1.3.3. Immunopathogenesis

Psoriasis is classified as an immune-mediated disease, characterised by a chronic and persistent inflammation. Typical alterations in psoriasis are hyperproliferation and abnormal differentiation of keratinocytes, lymphocyte infiltration consisting mostly of T lymphocytes, and various endothelial vascular changes in the dermal layer, such as angiogenesis and capillary dilatation. However the precise mechanism and sequence of interactions triggering the inflammatory cascade are still unknown. In the last years, different experimental findings and clinical observations have shown a central role for T cells in psoriasis pathogenesis. The first evidence supporting this idea came from the use of cyclosporine, a generalised T-cell immunosuppressant, whose administration to psoriatic patients led

to a remarkable improvement of the disease (Ellis, Gorsulowsky et al. 1986). Subsequently, administration of monoclonal antibodies or other specific approaches targeting T cells (Weinshenker, Bass et al. 1989; Prinz, Braun-Falco et al. 1991; Gottlieb, Gilleaudeau et al. 1995) confirmed the pivotal role of T lymphocytes in psoriasis. Moreover, experiments with immunodeficent mice transplanted with non involved skin from patients with psoriasis provided support for the concept of T cell mediated pathogenesis (Nickoloff and Wrone-Smith 1999). Finally, in the last years, it has been shown that tissue-resident immune cells in psoriasis may be sufficient to induce the epidermal changes typical of the disease (Boyman, Hefti et al. 2004; Boyman, Conrad et al. 2006). All these evidence are further supported by the fact that increased levels of activated T lymphocytes are present in psoriatic skin plaques and blood of patients (Bata-Csorgo, Hammerberg et al. 1995; Ellis and Krueger 2001).

1.3.3.1. Development of skin lesions and the local immune system

Although the initial event triggering a psoriatic lesion is still unknown, many environmental factors have been shown to play a role in the pathogenesis of psoriasis. External triggers such as physical trauma (known as the Köbner phenomenon), infections, stress, drugs, alcohol and smoking can all trigger an initial episode of psoriasis in those individuals who already have a genetic predisposition (Bowcock and Krueger 2005). These triggers also worsen the disease and can induce a severe relapse. Among all the external triggers mentioned above the only one which has consistently been associated with initiation of psoriasis is streptococcal infection. Group A streptococci have been related to the onset of psoriasis, especially acute guttate psoriasis, by inducing cutaneous lymphocyte associate antigen (CLA) expression on T cells, thereby facilitating T cell migration in the skin (Leung, Gately et al. 1995). It is also postulated that bacterial endotoxins can act as superantigens, activating T cells, macrophages and Langerhans cells (Swerlick, Cunningham et al. 1986). In fact streptococcal M proteins have epitopes common to some human keratins which are up-regulated in psoriatic lesions, but usually absent in healthy skin (Gudjonsson, Johnston et al. 2004). Hence psoriasis may be induced and exacerbated in susceptible individuals by M-protein specific Th1

cells that cross-react with human epidermal keratin (Valdimarsson, Sigmundsdottir et al. 1997).

Even if the real antigen which triggers psoriasis has yet to be identified, researchers highlight three key steps in the development of psoriatic plaque. The first event driving psoriatic inflammation is the migration of CLA+ activated T cell into the dermis. Progressive accumulation of leukocytes and progressive epidermal hyperplasia characterise the evolution to an acute lesion. During this step some T cells, DCs and neutrophils start to infiltrate the epidermis and release proinflammatory cytokines, which in turn stimulate keratinocytes inducing epidermal hyperplasia. An acute lesion rapidly evolves to a chronic plaque characterised by a continuous migration of cells, especially CD8+T cells, to the epidermis and the accumulation of mature DCs together with T cells around dermal blood vessels (Bowcock and Krueger 2005).

The events described above depict a multi-step process, which requires the recruitment of recirculating immune cells to the skin in order to develop a new psoriatic lesion. However, it is recently been proposed that once the very first episode inducing psoriasis has occurred, a subsequent inflammatory lesion can be triggered and sustained by the local skin immune system (Figure 1.1) (Boyman, Conrad et al. 2006). The evidence supporting this concept arises from the use of an established mouse model of psoriasis (Boyman, Hefti et al. 2004), which will only briefly be described in this section. Highly immunosopressed mice strains (AGR 129 mice, which lack both type I and type II IFN receptors and the recombination activating gene necessary for the immunoglobulin and T receptor gene recombination (RAG2-/-)) were transplanted with symptomless psoriatic skin of patient with psoriasis. Upon transplantation the grafts developed in typical psoriatic lesion, without requiring injection of activated T cells. The explanation for this phenomenon was that skin resident cells already present in the graft were necessary and sufficient for disease development. In fact, no T cells were found in the lymph nodes or in the spleen of grafted mice, showing that the cells present in the graft were sufficient to generate the lesion. Moreover the failure to generate a psoriatic lesion after administration of an anti-CD3 mAb, demonstrated that T cells and not KCs alone were necessary to generate the psoriatic phenotype. The theory that skin-resident immune cells are sufficient to maintain and re-induce a psoriatic lesion

during the chronic relapsing course of the disease, is reinforced by the work of Clark et al (Clark, Chong et al. 2006 24). They propose that the majority of skin-homing lymphocytes reside in normal skin and that there are twice so many cells resident in the skin than are present in the circulation. The majority of these cells are memory T cells that are Th1 polarized and involved in cutaneous immunosurvelliance. Considering these two studies into account the development of a psoriatic lesion could be explained as follows (Figure 1.4). Once a patient has developed his first psoriatic lesion, his symptomless skin may be activated in a much faster manner due to the strategic positioning of pDCs, dermal DCs and T cells. Thus, an innocuous trigger could activate the resident cells, eliciting the inflammation cascade typical of psoriatic lesion, without recruitment of cells form the recirculation. After the initial trigger, one of the earliest events driving the inflammatory eruption are the secretion of INF-α from pDCs and the production of TNF-α by cells of the innate and adaptive immune system. High amounts of IFN-α released by pDCs induces the activation of the local immune effector cells that become capable of secreting cytokines that further promote the inflammatory cascade. TNF-α is a highly active cytokine of the inflammatory infiltrate and is an important pro-inflammatory factor in the development of psoriasis. Its important role in the pathogenesis of this skin disease has been demonstrated by the efficacy of anti- TNF-α–therapies (Infliximab and Etanercept) in patient with psoriasis (Kleyn and Griffiths 2006). TNF-α is mainly released by activated macrophages, dermal DCs and in a lesser extent by KCs and T cells. The high amounts of this released cytokine have mainly two direct consequences in the skin. Firstly, TNF-α leads to the maturation of DCs into more powerful APCs, secondly, in conjunction with other cytokines TNF-α up-regulates the expression of endothelial E-selectin and ICAM-1 on skin vessels. In this way, more and more CLA+ T cells will extravasate into the skin and take part in the inflammatory process (Guenther and Ortonne 2002). The panel of cytokines released by T lymphocytes is also responsible for potent stimulation of epidermal keratinocytes. They trigger keratinocyte hyperproliferation and induce the expression of ICAM-1, CD40 and MHC-II, which favour the migration of T cells in the epidermis through LFA-1/ICAM-1 interactions.

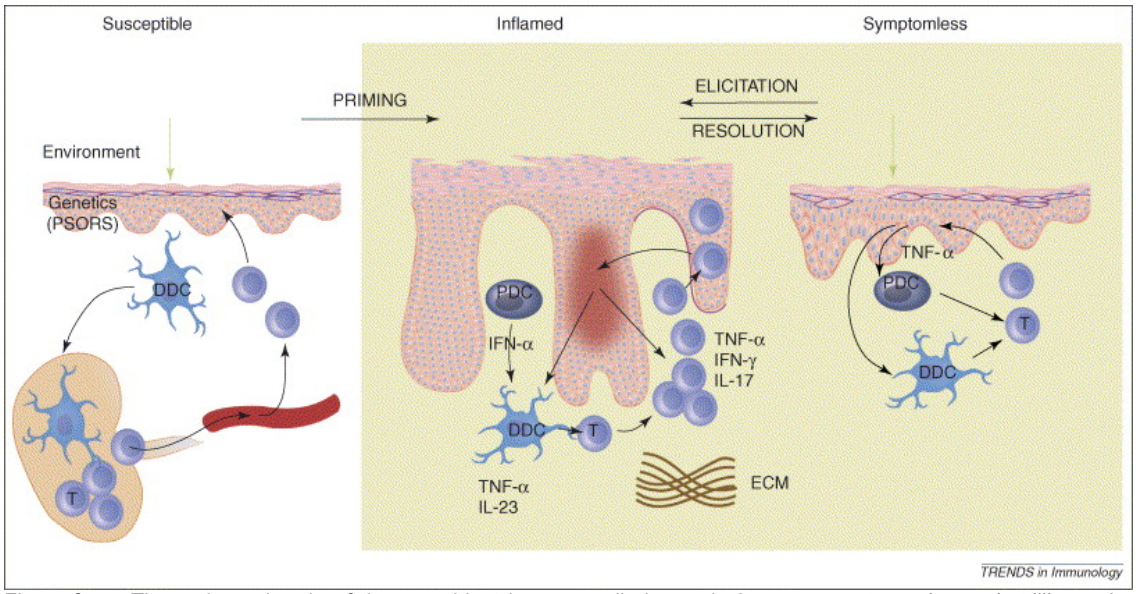

Figure from: The pathogenic role of tissue-resident immune cells in psoriasis. *Boyman, Conrad, Tonel, Gilliet and Nestle*, Trends in Immunol 2006

Figure 1.4 Local skin immune system in psoriasis. Psoriatic patients who have already experienced a psoriatic lesion may quickly develop a second one. Individuals with a genetic predisposition might develop psoriasis after an external trigger (physical trauma, infection, stress, etc.). During this first event (priming), dermal DCs (DDCs) migrate to the local lymph node and activate (auto)antigen-specific T cells (T). Subsequently, T cells migrate to the skin where they induce, together with PDCs and DDCs, the formation of a psoriatic skin lesion. After resolution of this inflamed state these patients might develop a second lesion more quickly (elicitation). The skin cells already present are necessary and sufficient to reinduce an inflamed state, without the requirement of recirculating cells.

Finally, the cytokine milieu seems to be also responsible of the characteristic vascular changes observed in the dermis of psoriatic lesions, such as dilatation and tortuosity of capillaries, angiogenesis and high endothelial venule (HEV) formation. Angiogenesis and hyperpermeabilty are also a direct consequence of the high levels of VEGF/VPF (vascular permeability factor) produced by keratinocytes (Guenther and Ortonne 2002).

From this scenario it becomes clear that cytokines play a central role in the initiation, maintenance and recurrence of psoriasis. Beside the already mentioned cytokines, IL-1, IL-2, IL-6, IL-8, IL-15, IL-22 and IL-23 have also been demonstrated to amplify the inflammatory reaction, which finally ends to the characteristic features of psoriasis.

1.3.3.2. T cells in chronic plaques

As mentioned before, the number of T cells present in psoriatic lesions is highly increased when compared to the skin of healthy individuals. Moreover, large number of lymphocytes populates the epidermis of a chronic psoriatic lesion, while in normal skin no T cells are found at this level. The majority of T lymphocytes localised in the dermis are CD4+ Th cells, while those migrating to the epidermis are mainly CD8+ Tc cells. The majority of T cells in psoriatic plaques are CD45RO+ subtype, indicating that they are memory T cells that migrate into skin in recognition of a yet undetermined antigen. Moreover, these cells are persistently activated T cells, as identified by the surface expression of activation-associated molecules CD69 and CD25 (Ferenczi, Burack et al. 2000). Some investigators have reported an oligoclonal expansion of CD8+ T cells in psoriatic lesions, suggesting that these T cells have been clonally expanded by an antigen present in the skin (Chang, Smith et al. 1994; Lin, Norris et al. 2001). However, no consistent data have been reported regarding TCR clonality and further studies have to be done in order to confirm the real presence of psoriasis-specific T cell clones.

Three other types of T cells might be important in psoriasis: interleukin 17 producing T cells (Th-17), Treg and NK T cells.

Th-17 cells are a recently identified lineage of effector CD4+T cells characterised by production of IL-17. Mouse models suggest that TGF-β and IL-6 mediate de novo differentiation of these cells from naïve precursors (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006). Survival and expansion of Th-17 cells on the other hand, seems to depend on IL-23, a cytokine produced by DCs and keratinocytes. IL-23 has been shown to have a critical role in the development of several classic autoimmune inflammatory diseases in mice (Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003). Recent studies have also shown that both IL-17 and IL-23 are increased in psoriatic skin (Teunissen, Koomen et al. 1998; Lee, Trepicchio et al. 2004). An interesting study by Sugiyama at al.(Sugiyama, Gyulai et al. 2005) showed that Treg cells are both functionally and numerically impaired in psoriasis, and as a result they cannot restrain the ongoing chronic inflammation. In this regard, it has been suggested that Treg and Th-17 may differentiate from the same precursor, depending on the balance of cytokines present in the surrounding milieu. Therefore, Treg and Th-17 cells may arise in a mutual fashion, depending whether they are

activated in the presence of TGF-β alone or TGF-β plus IL-6 respectively. In autoimmune diseases, as well as in psoriasis, the high amounts of IL-6 produced by the activated innate immune system would suppress the generation of Treg cells and induce a pro-inflammatory response dominated by Th-17 cells (Bettelli, Carrier et al. 2006). Whether this scenario is of relevance in human pathology, future studies will show.

Finally, there are few studies assessing the presence of NK T cells in plaques of psoriasis (Nickoloff, Bonish et al. 2000)(Cameron, Kirby et al. 2002). The exact function of these cells is still unclear, although they may inhibit or modulate inflammation as a reduced NK-T activity in animals appears to predispose to autoimmunity.

1.3.3.3. DCs in chronic plaques

Beside T cells, large amounts of DCs are also present in the psoriatic plaque. Both epidermal LC and dermal dendritic cells are increased in psoriatic skin lesions. Many of the dermal DCs display a mature or activated phenotype as identified by the expression of mature DC markers like CD80, CD83 and DC-LAMP. In contrast, few mature DCs are found in healthy skin or uninvolved psoriatic skin (Krueger and Bowcock 2005). Interestingly, Nestle at al showed that dermal DCs, derived from psoriatic plaque, strongly stimulated autologous T proliferation. Moreover they showed that these cells were six times more potent in inducing T cell proliferation compared to psoriatic blood-derived DCs or normal skin derived DCs (Nestle, Turka et al. 1994).

Beside dermal DCs and LCs, another subset of DCs has been identified in the dermal psoriatic skin. Plasmacytoide DCs (pDCs) are absent in the skin of healthy individuals, but it has been shown to be extremely increased in the skin of patients with psoriasis (Nestle, Conrad et al. 2005). The activation of these cells trough TLRs and the resulting secretion of IFN-α may activate immune T cells in psoriatic skin, leading to the development of new psoriatic lesions. Indeed, it has been shown that the high amounts of IFN-α released by pDCs can induce myeloid DCs activation and maturation, increasing the presentation of autoantigens to the local autoimmune T cells (Blanco, Palucka et al. 2001). Finally, high concentration of IFN-α could also be

responsible of the impaired migration of LCs shown in psoriasis, not attributable to the lack of cytokine signals (TNF-α and IL-1β) normally required for migration (Cumberbatch, Singh et al. 2006).

1.3.3.4. Keratinocytes in psoriasis

Over the last 20 years it has been continuously discussed whether psoriatic skin lesions arise from a primary alteration in epidermal KCs or in immunocytes. As mentioned before the current dogma is that infiltrating T cells initiate and maintain psoriasis. In this view, cytokines released by T cells and other inflammatory components (as DCs, macrophages and neutrophils) would trigger KC hyperproliferation. Some inflammatory cytokines (e.g. IL-1, IL-6 and IFN-γ) have been shown to directly induce epidermal hyperplasia (Krueger 2002). Recently, IL-23, whose expression is increased in psoriatic lesion compared to healthy and psoriatic non-lesional skin (Piskin, Sylva-Steenland et al. 2006), has been implied in the development of epidermal acanthosis, most likely through the induction of IL-22 (Chan, Blumenschein et al. 2006; Zheng, Danilenko et al. 2006). Injection of this cytokine into mouse ear or into the dermis induces epidermal hyperplasia and dermal inflammation similar to features seen in psoriasis (Chan, Blumenschein et al. 2006). Acanthosis may be a direct consequence of the observed increase of KCs STAT-3 (signal transduction and activation of transcription 3), a pathway already shown to be critical for the development of psoriasis (Sano, Chan et al. 2005). An additional explanation for the chronic epidermal hyperplasia in psoriatic lesions has been suggested by Krueger (Krueger 2002), referring to an unpublished study done together with L Austin. The migration of T cells in the epidermis would first break the basement membrane, which has been shown to have large gaps or areas with reduced staining intensity for collagen IV and laminins (Fleischmajer, Kuroda et al. 2000), and secondly disrupt desmosome connection between KCs. These two events could be interpreted by KCs as an injury and therefore induce a wound repair response. As a consequence a lot of mitogenic cytokines would be released by KCs triggering a regenerative epidermal growth. Hence, T lymphocytes in psoriatic epidermis would be responsible for the chronic hyperplasia both by releasing pro-inflammatory cytokines and by disrupting epidermal integrity.

However, other studies are highlighting the central role of KCs in the induction of psoriasis. From the histological aspect, it has been claimed that psoriasis does not look like a T-cell skin disease, as i.e. lichen planus or lupus erythematosus (Nickoloff, Schroder et al. 2000), and also that the Koebner phenomenon can not be explained only by T cell aetiology. Moreover, some drugs that are used to treat psoriasis, e.g. cyclosporine A, not only interact with T cells activity, but also inhibit KC proliferation. Another evidence against the fundamental role of T cells in psoriasis arise from the observation that patient infected by HIV (human immunodeficiency virus), who have a reduced number of CD4+T cells, develop psoriasis with the same frequency as the rest of the population. However, patients with HIV infection often present with more severe clinical manifestations of psoriasis than the non-HIV population. (Namazi 2004). Finally, in 2005 Zenz and al. reported an interesting animal model with psoriasis-like features (Zenz, Eferl et al. 2005). The authors knocked out JunB and c-Jun, two components of the AP-1 transcription factor, which control cell proliferation and differentiation, cytokine production, and stress responses in the skin. Interestingly, these mice developed not only inflammatory skin lesions but also a form of destructive arthritis. This evidence suggested that alteration in the epidermal Jun-pathway may be sufficient to induce inflammatory reactions in the skin as well as in the joints. At the skin level these mice showed typical hallmarks of psoriasis: hyperkeratosis, enlarged blood vessels, infiltration of CD3+ cells and neutrophilis, as well as upregulation of several cytokines. Finally, the importance of this animal model lies in the fact that JunB is located in the PSORS6 locus, which has been shown to be a psoriasis susceptibility region on chromosome 19 (Hensen, Windemuth et al. 2003). The above described transgenic animal model shifted again the interest towards KCs as first initiator in psoriasis. However, in order to confirm this hypothesis a better characterization of this mice model is required. In this context Brian Nickoloff (Nickoloff 2006) has raised some interesting questions with respect to this new animal model. Firstly, he argues if the epidermal thickening seen in the animal model of Zenz et al (Zenz, Eferl et al. 2005) can really be classified as a psoriatic lesion or could it be also attributed to other skin inflammatory disorders, such as atopic dermatitis. In fact there are a lot of human skin diseases in which epidermal hyperplasia is a consequence of a wound healing process. Secondly, even if in this model T cells were not necessary for disease development, Nickoloff believes that a better characterization of the CD3+ cell population in this psoriasis model is

mandatory. Nickoloffs observations in turn imply that before using animal models to study psoriasis rigorous testing is required in order to conclude that the model of interest truly resembles this unique human skin disease.

1.3.4. Animal models of psoriasis

Psoriasis seems to be a uniquely human disease (Conrad and Nestle 2006). Therefore, due to the lack of a natural occurring animal model, most of the research regarding the pathogenesis of psoriasis has been done on transgenic and xenotransplantation animal models. Through these models the pathogenic function of several cytokines and genes could be defined as well new insights into the immunology of psoriasis could be gained.

Table 1.2 Animals models for psoriasis

MODEL	*EPIDERMAL HYPER PROLIFERATION*	*INCREASED VASCULARITY*	*INFLAMMATORY CELLS*	*T CELL INFILTRATE*	*ANTIPSORIATIC TREATMENT*	*REFLECTS HUMAN SITUATION*
Transgenic mice						
K14/KGF K14/IL-6 K14/amphiregulin	Yes	Yes	Yes	No	No data	No
K14/VEGF	Yes	Yes	Yes	No data	No data	No
K14/IL-1α K14//TGF-α	Yes	No data	Yes	Few animals	No data	No
Involucrin/IFN-γ	Yes	Yes	Yes	Yes, not epidermal	No data	No
STAT-3C	Yes	Yes	Yes	Yes	No data	Yes
Knockout mice						
Epidermal IKK2	Yes	Yes	Yes	Yes, no functional role	No data	No
Epidermal Junb/c-jun	Yes	Yes	Yes	Yes, marginal role	No data	No
Xenotransplantation						
SCID	Yes	Yes	Yes	Yes	Yes	Yes
AGR129	Yes	Yes	Yes	Yes	Yes	Yes

Adaptation from Conrad and Nestle (Conrad and Nestle 2006)

Different inflammatory mediators or growth factors, which have been proposed to participate in the development of psoriasis, have been overexpressed or knocked-out in order to make a genetically modified mouse model resembling this skin disease (Table II). For example, transgenic mice with epidermal overexpression of IL-1α (K14/IL-1α) developed a spontaneous skin disease characterised by scaling and focal inflammatory skin lesions (Groves, Mizutani et al. 1995). Lesional skin of these animals presented histologically with a mixed cellular infiltrate, acanthosis and

parakeratosis. The effect on keratinocyte differentiation appeared to be modest, indicating that there are also other factors in psoriatic lesion that were absent in this animal model. On the other hand the IL-1α transgenic animal model showed a primary role of this cytokine during cutaneous inflammation. In order to study the role of IFN-γ in the pathogenesis of inflammatory skin disease, Carroll at al created a transgenic mice overexpressing IFN-γ in the superbasal layers of the epidermis (Carroll, Crompton et al. 1997). The skin of affected mice was characterised by keratinocyte hyperproliferation, enlarged dermal capillaries and dermal infiltration consisting of T cells and monocytes. The absence of T cells and LCs in the epidermis suggested that IFN-γ does not influence the migration of cells as seen in human psoriasis. Taken together, all these transgenic mice models fail to generate skin lesions with all the hallmarks of psoriasis, suggesting that a single cytokines is apparently not sufficient to induce the full phenotypic appearance of the disease.

In recent years, some new genetically modified mice models, showing many characteristic features of psoriasis, have been published. Sano et al. generated a mouse model that constitutively overexpressed activated STAT-3 in the basal stem-cells of the epidermis (Sano, Chan et al. 2005). Within two weeks after birth, animals developed reddened, scaling, thickened skin lesions on the tail and paws. Lesions progressed overtime to the trunk. Histolotgically the lesions showed a marked hyperplasia of the epidermis, loss of the granular layer, inflammatory infiltrates and angiogenesis. Furthermore, by blocking STAT-3 with a decoy oligonucleotide (which competes for binding of STAT-3 and interferes with its transcriptional activity) the disease could no longer progress. Finally, the authors showed the presence of activated STAT-3 in keratinocytes of uninvolved skin of psoriatic patients, implying pivotal role of this pathway in the development of psoriasis. Another important observation is that the development of psoriatic lesions required not only STAT-3 activation in keratinocytes but also the presence of activated T cells. These data suggests that the development of a full psoriatic lesion may depend from the co-presence of a signalling defect in keratinocytes and an inappropriate immunocytes activation state (Nickoloff 2006).

Zenz and colleagues developed another interesting new mouse model by double-knockout JunB and c-Jun (Zenz, Eferl et al. 2005). JunB, belongs to a family of transcription factors and is known to regulate cell proliferation, differentiation and

stress responses (Zenz, Eferl et al. 2005). Its activity is greatly reduced in lesional psoriatc skin, however, mice lacking JunB did not show any signs of psoriasis. Only through the combined deletion of JunB and c-Jun, a closely related gene proposed to antagonise JunB function (Zenz, Eferl et al. 2005), the typical symptoms of the disease could be induced. Animals mutated for JunB/c-Jun developed scaly plaques on their ears, paws and tails that closely resembled the characteristic lesions of human psoriasis. On histological examination, the epidermis was thickened and had prominent rete ridges, with hyperkeratosis and parakeratosis in the upper layers. Dermal vascularization was increased. Strictly the mice also developed arthritic lesions, with periostitis and massive bone destruction, similar to the psoriatic arthritis seen in humans. Interestingly, the deletion of epidermal JunB and c-jun in Rag2-deficeint mice (mice lacking T and B cells) still led to the development of skin lesions, though they were milder than those in the mice with normal Rag2 activity, suggesting that T and B cells make a minor contribution to the development of skin symptoms in psoriasis. In these Rag2-knockout mice joint inflammation and bone destruction were strongly reduced, leading to the conclusion that functional immune cells are necessary for the development of psoriatic arthritis (Zenz, Eferl et al. 2005) but not skin inflammation. However, conflicting data about the role of JunB in psoriasis are derived from another study showing that mRNA and protein levels of JunB were increased and not reduced in psoriatic lesions (Haider, Duculan et al. 2006). Moreover, this study showed that genes depending on the transcriptional activation of JunB were up-regulated, showing that JunB was biologically active in psoriatic lesions.

Compared to transgenic mouse models, xenotransplantation models for psoriasis are very attractive because of their relatedness to human situation. Initially, investigators focused on human skin grafted onto nude mice. Using this approach, psoriasis could be maintained for more then 2 months without further manipulations (Fraki, Briggaman et al. 1983). Subsequently, uninvolved psoriatic skin was engrafted onto SCID (severel combined immunodeficiency disease) mice, which cannot make T and B cells but retain the capacity to produce neutrophils and NK cells. Therefore, they have been extensively used as host for tissue transplants. By using this immunodeficient animal model the pathogenic link between T cells isolated from psoriatic patients and the induction of skin alterations characteristic of psoriasis

has been demonstrated (Nickoloff, Schroder et al. 2000). Basically, when T cells from psoriatic patients were activated (using different stimuli such as bacterial superantigens, IL-2, etc) and injected into non involved psoriatic skin, consistent phenotypic conversion to a psoriatic plaque lesion was observed. Interestingly, only lymphocytes from psoriatic patients could promote plaque formation, and not immunocytes from healthy individuals. Moreover, only CD4+ T cells, but not CD8+, cells were able to induce psoriatic plaques formation (Nickoloff and Wrone-Smith 1999). The mentioned animal model has also been successfully used as an experimental tool for preclinical screening of potential anti-psoriatic treatments (Nickoloff and Nestle 2004). Besides the SCID mice another new psoriasis xenogenic model has been evaluated recently (Boyman, Hefti et al. 2004). In this new approach, AGR129 mice were used as acceptor of human skin grafts. AGR129 mice have no functional receptors for type I (IFN-α/β) and type II IFN (IFN-γ) and therefore show immature NK cells with impaired cytotoxic activity. Moreover they carry a third deletion (RAG) that enables the production of T and B cells. Upon transplantation on these mice, uninvolved psoriatic skin grafts spontaneously converted to psoriatic lesion without injection of any exogenous factor. Such spontaneous development of a full psoriatic phenotype indicates that the local environment present in the symptomless psoriatic skin is sufficient to drive psoriasis plaque formation. By using this new psoriasis mouse model new insights into the immunopathogenesis of psoriasis have been provided. Firstly, the model highlighted the importance of resident T cells for the development of the psoriatic lesion as by treating engrafted skin with anti-CD3 antibodies or TNF-α inhibitors the conversion into a psoriatic phenotype could be prevented. Secondly, this model showed that during development of psoriatic lesion T cell proliferation takes place in the dermis that is followed by a decrease of T cells in the dermis and a concurrent increase of T cells in the epidermis (Boyman, Hefti et al. 2004).

A recent study has demonstrated that pDCs infiltrate uninvolved skin of psoriatic patients and become activated to produce IFN-α in the early phase of the disease development (Nestle, Conrad et al. 2005). Consequently, pDC-derived IFN-α would activate and expand the pathogenic T cell population already present in the skin, causing formation of psoriatic skin lesions. When the production of INF-α was blocked or prevented, the development of psoriasis was likewise blocked. This study

provided the first evidence for a role of pDCs-derived IFN-α in the pathogenesis of an autoimmune disease and presented IFN-α as a key cytokine in the development of psoriasis. The presented mouse model is the first in which psoriasis appeared spontaneously after skin engraftment and which allows the study of very early events leading to the development of psoriasis. However, there are limitations in the use of xenografts models.Upon transplantation no human bone marrow derived cells can participate in lesion formation, which precludes studies of trafficking between circulating cells and skin.

1.4. Overview

This thesis sought to delve deeper into the immunopathogenesis of psoriasis. In particular this work shows that cytokines, T cells and integrins are essential elements of psoriatic lesion formation. Chapter 2 provides functional evidence for a crucial role of the IL-23 axis in cutaneous inflammation and demonstrates the beneft of blocking the p19 subunit of IL-23 in the treatment of psoriasis. Chapter 3 provides evidences that although IL-17-producing cells take part in the inflammatory reaction during the formation of a psoriatic lesion, the inhibition of IL-17 function is not sufficient to block the onset of disease.. The first part of Chapter 4 is taken from an article published in Nature Medicine (Conrad, Boyman et al. 2007). This part shows the importance of extracellular matrix/T cell interactions in psoriasis and demonstrates that collagen IV binding integrin $\alpha_1\beta_1$ is essential for expansion of epidermal T cells and the manifestation of the disease. The second part of chapter 4 focuses on the role of another integrin, $\alpha_2\beta_1$, during psoriasis onset. It demonstrates that interaction of $\alpha_2\beta_1$ with collagen I causes KCs and ECs hyperproliferation and presents a T-cell independent mechanism for inhibiting the development of psoriatic lesions. Chapter 5 contains concluding remarks of the data presented throughout this thesis work, outlines possible future experiments and opens further questions in the field of psoriasis research.

Chapter 2

IL-23 promotes the development of new psoriatic skin lesions

2. IL-23 promotes the development of new psoriatic skin lesions

2.1. Introduction

The heterodimeric cytokine IL-12 promotes IFN-γ production by NK cells and thereby resistance to intracellular infections, especially bacteria, protozoa and fungi. It also induces the differentiation of IFN-γ-secreting Th1 cells and has been implicated in chronic inflammatory disorders characterised by excessive Th1 response (Trinchieri, Pflanz et al. 2003). In 2000, Kastelein and Bazan identified the new p19 subunit and showed that it dimerized with IL-12p40, to form a cytokine that they called IL-23 (Oppmann, Lesley et al. 2000). IL-12 and IL-23 belong to the IL-12 family of heterodimeric cytokines and are composed of the identical p40 subunit plus a unique p35 and p19 subunit respectively. IL-12 and IL-23 receptors share the common IL-12Rβ1 (IL-12 receptor beta 1), for binding of p40, but require IL-12β2 and IL-23R respectively to induce signal transduction. These two cytokines activate a JAK-STAT (Janus kinase/signal transducer and activator of transcription) signal transduction cascade, which subsequently induces the activation of target genes in the nucleus (Trinchieri, Pflanz et al. 2003). The main difference between the signalling pathways of IL-12 and IL-23 is that IL-12 leads to the phosphorylation and activation of STAT-4 homodimers, whereas IL-23 mainly induces the formation of STAT-3/STAT-4 heterodimers (Figure 2.1). DCs and monocytes/macrophages are the major producers of these two cytokines (Trinchieri, Pflanz et al. 2003). IL-23p19, in the same manner as the p35 subunit of IL-12, is poorly secreted until the same cell produces the p40 subunit. An explanation for this tightly regulated secretion may be that an aberrant release can lead to severe inflammation (Hunter 2005). In this context it has been shown that mice ubiquitously overexpressing IL-23p19 develop systemic inflammation and die prematurely due to impaired growth (Wiekowski, Leach et al. 2001). Similarly, a constitutive overexpression of p40 in basal keratinocytes leads to the development of cutaneous inflammation with the characteristic of an eczematous skin disease (Kopp, Kieffer et al. 2001)(Kopp, Lenz et al. 2003).

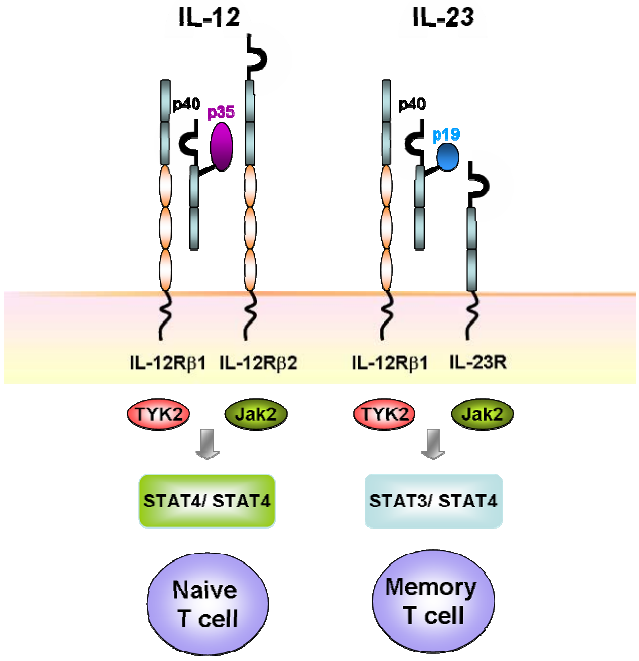

Figure 2.1 IL-12/IL-23 structure and signal transduction. IL-12/IL-23 are covalently linked heterodimers composed of a unique light chain (IL-12p35/IL-23p19) and a common heavy chain (IL-12p40). IL-12Rβ1 heterodimerizes with IL-12β2 to form the IL-12 receptor, whereas the dimerization of IL-12Rβ1 with IL-23R creates the IL-23 receptor. Engagement of these two cytokines with their receptor activate a similar spectrum of JAK-STAT molecules. IL-12 preferentially induces STAT-4 homodimers, whereas IL-23 induces STAT-3/ STAT-4 heterodimers. Through activation of STAT-4 the primary action of IL-12 is to induce differentiation of naïve T cells and the production of IFN-γ, while IL-23 may support the proliferation of memory T cells and the secretion of IL-17.

The receptor complexes for IL-12 and IL-23 are primarily expressed on T and NK cells, but also on DCs and macrophages suggesting that these cytokines also act in an autocrine manner (Bastos, Marinho et al. 2004). Due to their structural similarities it was expected that IL-12 and IL-23 have overlapping activities. However, several studies published in the last years have shown that these two cytokines show very distinct functions. IL-12 induces the production of IFN-γ from T and NK cells and promotes the differentiation of naïve T cells into Th1 effector cells. Whereas IL-23 acts on CD4+ memory T cells and promotes the expansion and survival of previously differentiated Th-17 cells (Bettelli, Oukka et al. 2007). In the last years it has also been demonstrated that chronic inflammatory diseases, especially in mice, previously attributed to IL-12 (Leonard, Waldburger et al. 1995)(Malfait, Butler et al. 1998), are indeed caused by IL-23. Perhaps the most compelling evidence for the fundamental role of IL-23 in the development of autoimmune disorders derives from studies done in the experimental autoimmune encephalomyelitis model (EAE). Cua et al, by using gene-targeted mice lacking only IL-23 and cytokine replacement studies, were the

first to demonstrate that IL-23, but not IL-12, is the critical cytokine in this disorder (Cua, Sherlock et al. 2003). In this work the authors compared the induction of EAE in mice lacking only either IL-23 ($p19^{-/-}$) or IL-12 ($p35^{-/-}$), or both cytokines ($p40^{-/-}$). After injection of the encephalitogenic myelin oligodendrocyte glycoprotein (MOG) only $p19^{-/-}$ and $p40^{-/-}$ mice were resistant to EAE. Moreover, the delivery of IL-23 into the central nervous system (CNS) reconstituted EAE in both $p19^{-/-}$ and $p40^{-/-}$ mice. The mentioned study demonstrates that during disease onset IL-12 promotes the development of naïve T cells, whereas IL-23 mediates late stage inflammation by activating memory T cells and macrophages to produce pro-inflammatory cytokines. Consistent with the dominant role for IL-23 in the CNS inflammation, further studies demonstrated that this cytokine is an essential mediator also for joint autoimmune inflammation (CIA) (Murphy, Langrish et al. 2003) and inflammatory bowel disease (IBD) (Kullberg, Jankovic et al. 2006).

In psoriasis, as in the disease mentioned before, IL-12 was thought to be the driving force behind the persistent inflammatory reactions. MRNA for the p40 subunit of IL-12 as well as expression of IL-12p70 protein was shown to be increased in psoriatic lesions as compared with normal and non lesional psoriatic skin (Yawalkar, Karlen et al. 1998). Moreover, the administration of an antibody against IL-12 in a psoriasis mouse model inhibited the development of the disease (Hong, Chu et al. 1999). However, with the identification of IL-23 and its role in many autoimmune diseases researchers argued for a possible role of this cytokine also in psoriasis. The first who confirmed this hypothesis were Lee and co-workers who showed a consistent increase in IL-23 production in psoriatic lesional skin, as compared to non involved psoriatic skin or skin from healthy volunteers. They showed a strong upregulation of both p40 and p19 subunits, but not of p35, indicating that IL-23 rather then IL-12 may be involved in the pathogenesis of psoriasis (Lee, Trepicchio et al. 2004). A subsequent study demonstrated that not only DCs and macrophages can produce IL-23, but also human keratinocytes constitutively express the p40 and p19 subunits of IL-23. Moreover, the keratinocyte-secreted IL-23 was shown to be biologically active and capable to induce IFN-γ production in memory T cells (Piskin, Sylva-Steenland et al. 2006). Recently, two independent studies demonstrated that intradermal injection of IL-23 induced the development of epidermal acanthosis in mice (Zheng, Danilenko et al. 2006)(Chan, Blumenschein et al. 2006). Additionally,

Zheng et al demonstrated that also IL-12 injection led to an increase in epidermal thickening, however at a much lower level then the one seen with IL-23. The infiltrating lymphocytes of both IL-12 and IL-23 treated mice were composed by CD4+ and CD8+ cells, but the two treatments induced different cytokines profiles. The injection of IL-12 induced an increase only of IFN-γ levels, whereas IL-23 led to the production also of IL-17 and IL-22, which were already shown to be increased in psoriatic lesions (Chan, Blumenschein et al. 2006)(Wolk, Kunz et al. 2004). Moreover, binding of IL-22 to its receptor, whose expression in the skin is confined to KCs, mediates epidermal acanthosis through the activation of STAT-3. These observations potentially explain the increased STAT-3 expression in the epidermal compartment of the previous mentioned IL-23 treated mice (Zheng, Danilenko et al. 2006) and in the skin of psoriatic patients (Sano, Chan et al. 2005).

Recent studies have supplied genetic evidence that the IL-23 pathway may be critical for the pathogenesis of psoriasis. The identification of two psoriasis-susceptibility genes, i.e. variants of IL-12B and IL-23R associated with psoriasis, underscore the importance of this pathway (Capon, Di Meglio et al. 2007; Cargill, Schrodi et al. 2007). The same variant in the IL-23R has been observed in IBD (Duerr, Taylor et al. 2006), suggesting that the IL-23R locus may play a key role in the development of chronic inflammatory diseases.

Promising data about the relevance of IL-23 in the immunology of psoriasis also come from a clinical study using a neutralizing antibody to IL-12p40 in patients with moderate to severe psoriasis vulgaris. Phase-I and -II studies showed the safety and the prolonged efficacy of a single intravenous injection of the human neutralizing antibody to IL-12p40 and demonstrated a concentration dependent efficacy in the treatment of psoriatic lesions (Kauffman, Aria et al. 2004; Krueger, Langley et al. 2007). Additional studies will define the potential of this compound in psoriasis. Nevertheless all these data together show the importance of studying the IL-23 pathway in psoriasis and provide the evidence that IL-23 or downstream cytokines may be a good target for psoriasis treatment.

The studies mentioned above showed a dysregulation of the IL-23 axis in lesional skin of patients with psoriasis and unequivocally demonstrated that the direct injection of this cytokine into murine skin induces dermal inflammation and

acanthosis resembling psoriasis. However, the functional role of IL-23 in human psoriatic lesions remains to be determined.

To better understand the function of IL-23 in the pathogenesis of this chronic inflammatory skin disorder the following experiments were performed. First, analyses were done on blood and tissues of psoriasis patients. A statistically significant increase of the IL-23 receptor was seen in peripheral CD3+ cells of psoriasis patients compared to healthy controls. In contrast, peripheral DCs and B cells of these two groups had similar levels of IL-23R expression. The next step was to determine the expression of IL-23R in the skin. In line with the results obtained in the blood with immune cell subpopulations, overexpression of IL-23R was detected on T cells and also on DCs of psoriatic patients compared to healthy donors. When IL-23R expression was analysed on the surface of KCs a similar pattern of expression was seen on cells isolated from the skin of psoriatic patients compared to that of healthy individuals. Subsequently, lesional psoriatic skin sections were stained with a monoclonal antibody specific for IL-23. Dendritic appearing cells in the dermis of psoriasis stained positive for this antibody, indicating that DCs are the main producer of IL-23 in this disease, as already shown by other groups (Lee, Trepicchio et al. 2004). However, in accordance with previous data presented by Piskins et al. (Piskin, Sylva-Steenland et al. 2006), further analyses showed the presence of IL-23 also in the epidermis of psoriatic lesion, indicating that also KCs are able to produce this cytokine. Finally, in order to put the tissue expression data in a functional context the role of IL-23 was analysed in a humanized psoriasis mouse model (the AGR xenotransplantation psoriasis mouse model). Administration of a neutralizing Ab against human IL-23 inhibited psoriasis development in a manner comparable to the use of anti-TNF-α blockers, the current “gold standards” of anti-psoriatic therapy. Anti-IL-23 monoclonal Ab (mAb) treatment also blocked human epidermal and dermal T cell expansion, typically seen during psoriasis development. In a final step, IL-23 kinetics during development of psoriatic lesions was monitored. This analysis showed that the appearance of IL-23 correlated with the increase of T cells in the skin and with epidermal hyperplasia during development of a psoriatic lesion

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2.2. Results

2.2.1. Analysis of IL-23R expression in the blood and in the skin of psoriatic patients

An IL-23R variant has been found to be associated with psoriasis in whole genome association studies (Capon, Di Meglio et al. 2007; Cargill, Schrodi et al. 2007). Specifically, the allele for the R381Q single nucleotide polymorphism conferred protection against psoriasis. The same variation has also been reported to strongly associate with Crohn's disease (Duerr, Taylor et al. 2006), suggesting that common genetic variants may contribute to the pathogenesis of chronic epithelial disorders, such as Crohn's disease and psoriasis. It is unknown whether IL-23R is differently expressed in psoriatic patient compared to healthy donors. The aim of the first part of this study was to analysed the IL-23R expression in the blood and in the skin of psoriatic patient and compare it with that of healthy volunteers.

2.2.1.1. Increased expression of IL-23R on psoriatic blood T cells

IL-23R+ PBMCs of psoriatic patients (n=12) and healthy individuals (n=13) were detected using a specific antibody against the IL-23R chain and subsequently analysed by flow cytometry (Figure 2.2C). T cells of patient with psoriasis showed higher levels of IL-23R expression compared to healthy donor's lymphocytes. A more detailed analysis of T cell subpopulations demonstrated that there was a statistically significant increase in CD3+ and CD4+/IL23R+ T cells in psoriatic patients compared to healthy controls (Figure 2.2A). In contrast, psoriatic CD8+ T cells had only a slightly increased expression of the IL-23 receptor when compared to the same subpopulation in the blood of healthy donors. DCs, identified as CD11c+/HLA-DR+ cells, were also expressing the IL-23R, but no statistical significance was seen in the two analysed groups. Three colour staining with IL-23R, CD19 and CD40 revealed that B cells also express the receptor for IL-23, but only a slight increase was seen by comparing these cells in psoriatic patients versus healthy donors (Figure 2.2B).

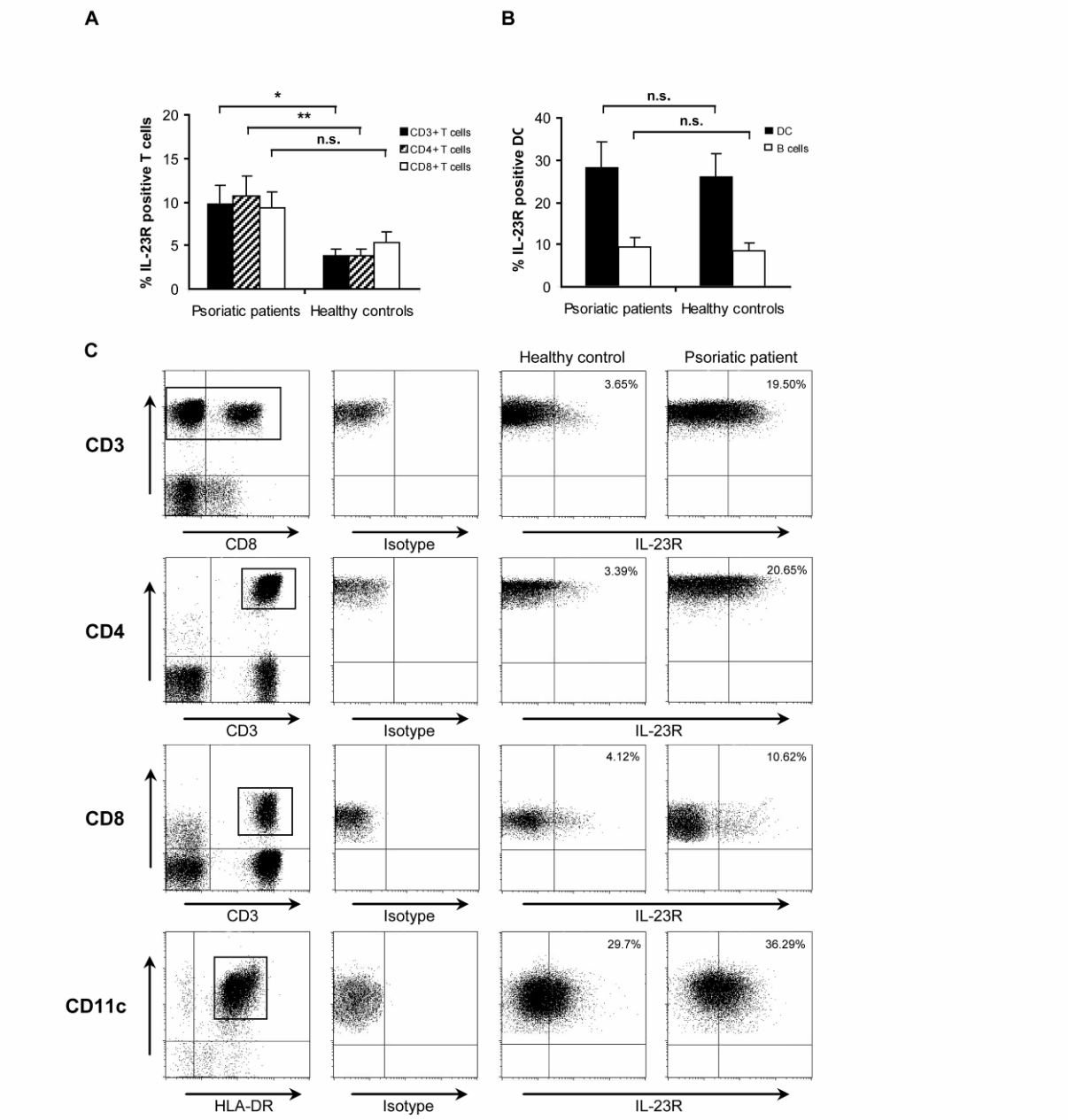

Figure 2.2 Increased interleukin-23 receptor expression in psoriatic blood cells. Expression of IL-23R on blood T cells of psoriatic patients and healthy controls (A). IL-23R expression showed a statistically significant increase on CD3+ (p=0.02) and CD4+ (p=0.01) T cells of psoriatic patients compared to CD3+ and CD4+ T cells of healthy controls. (B) IL-23R expression on DC and B cells. No significant difference was seen in the expression of IL-23R, neither for DCs or B cells, on psoriatic patients compared to healthy controls. (C) Examples of expression of IL-23R on cells freshly isolated from blood of healthy controls or psoriasis patients. The antibodies used for analysis are indicated on the x and y axes. Third and fourth column represent IL-23R expression in healthy controls or psoriatic patients respectively. The numbers in quadrants indicate percentage of cells staining positive for IL-23R. Dot plot shown are gated either on lymphocytes or DCs. Data represent mean of 12 (psoriatic blood) or 13 (healthy donors) respectively experiments performed on different donors.
* p= 0.02, ** p=0.01

2.2.1.2. IL-23R expression in lesional psoriatic skin

Experiments shown in paragraph 2.2.1.1 demonstrated that peripheral T cells of psoriatic patients had a significant increase of IL-23R compared to the same subpopulation in the blood of healthy donors. In contrast, only a slight increase in the level of IL-23R expression was seen in DCs and B cells of patients with psoriasis compared to healthy individuals. The next step was to investigate the expression of this receptor directly in the skin. For this purpose three biopsies of patients with chronic-plaque –type psoriasis and three skin samples from healthy volunteers (NN skin) were analysed. Split-thickness skin was obtained using a dermatome. Epidermal and dermal sheets were separated and cell suspensions, obtained after enzymatic treatment of epidermal or dermal sheets, were afterwards used for cytofluorimetric analysis. Psoriatic tissue T cells showed a significantly higher level of IL-23R expression compared to lymphocytes isolated from the skin of healthy individuals (Figure 2.3A). CD8+ lymphocytes had an increase, although not significant, of the receptor for IL-23. In contrast CD4+ T cells of psoriatic patients had similar expression of IL-23R compared to CD4+ cells isolated from the skin of healthy individuals. An explanation for these results could be that during separation of epidermal/dermal sheets an enzymatic step is required. Some enzymes, such as trypsin, have a proteolytic effect on CD4 (Lynch, Slaytor et al. 2003), which could explain the low levels of CD4+ cells found in the biopsies and also the few CD4/IL-23R double positive T cells present in both PP and NN skin. Upon examination of IL-23R on epidermal Langerhans cells (LCs) and dermal DCs of healthy individuals a lower expression level was observed when compared to the same cell population present in the tissue of patient with psoriasis (Figure 2.3B). The final part of this study focused on the expression of IL-23R on KCs, the main cells of the skin epidermis. KCs isolated from the skin of psoriatic patients (n=3) showed a similar pattern of expression to that observed in the epidermal cell suspensions of healthy individuals (n=3) (Figure 2.4). These data suggest that KCs constitutively express the IL-23R on their surface and that they do not increase the levels of this receptor in response to the inflammatory environment of a psoriatic lesion.

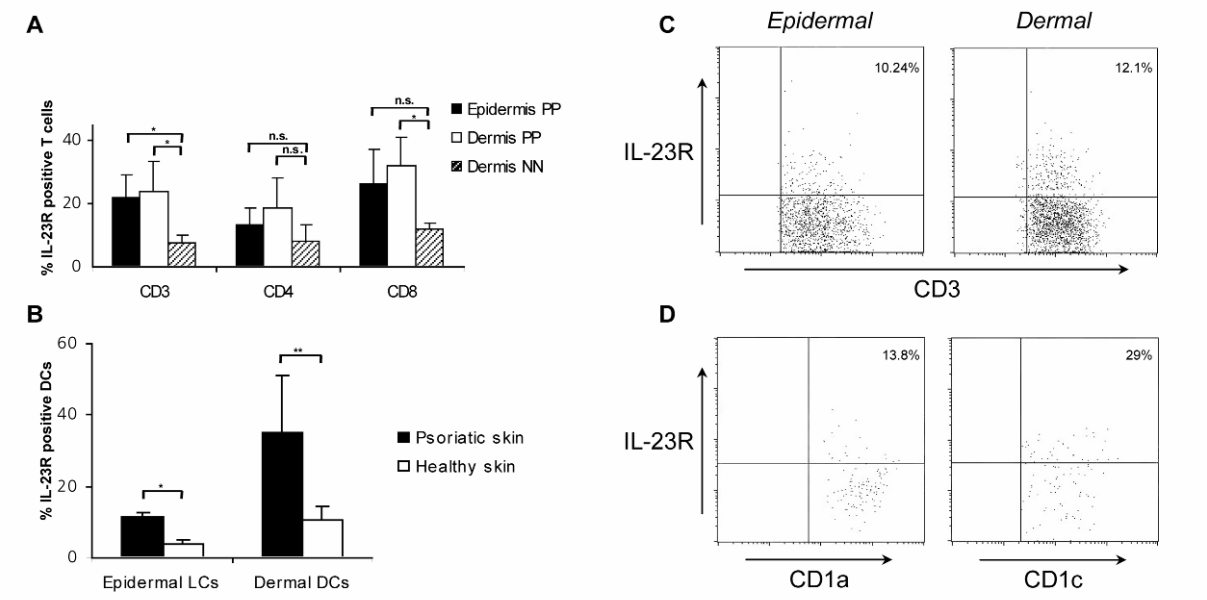

Figure 2.3 Expression of IL-23R in psoriatic and normal human skin. Significant increased levels of IL-23R expression were seen in epidermal and dermal psoriatic CD3+ (p=0.03) and CD8+ (p=0.03) cells when compared to CD3+ and CD8+ lymphocytes of healthy individuals (A). Dermal DCs and epidermal LCs isolated from NN skin presented significant less IL-23R levels on their surface compared to the same cells in the skin of psoriatic patients (B). Representative example of IL-23R expression on cells freshly isolated from the epidermis and dermis of psoriatic skin biopsies (C-D). Flow cytometry analysis revealed that approximately 12% of T cells in the epidermis and in the dermis as well were positive for IL-23R (C). Among the LCs in the epidermis and DCs in the dermis 14% and 30% respectively stained positive for the IL-23R (D). Graph C-D: Epidermal/dermal T cells and DCs data are gained from 2 distinct patients and are representative of three independent experiments. * p≤0.03, ** p=0.009

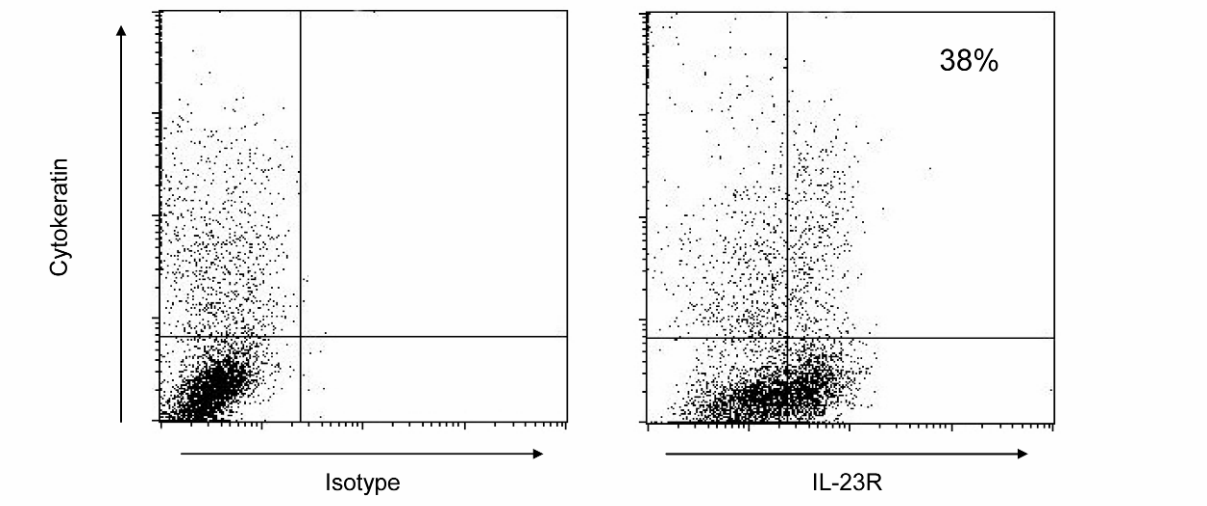

Figure 2.4 IL-23R expression in keratinocytes. Representative flow cytometry analysis of IL-23R expression in epidermal cell suspension isolated from psoriatic plaque lesions. Percentage of IL-23R+ KCs is indicated in the plot.

2.2.1.3. Identification of IL-23 expressing cells

Immunohistochemical stainings of skin sections done by Schering-Plough Biopharma showed that the IL-23 protein was localized in dendritic appearing cells in the dermis of psoriasis (Figure 2.5).

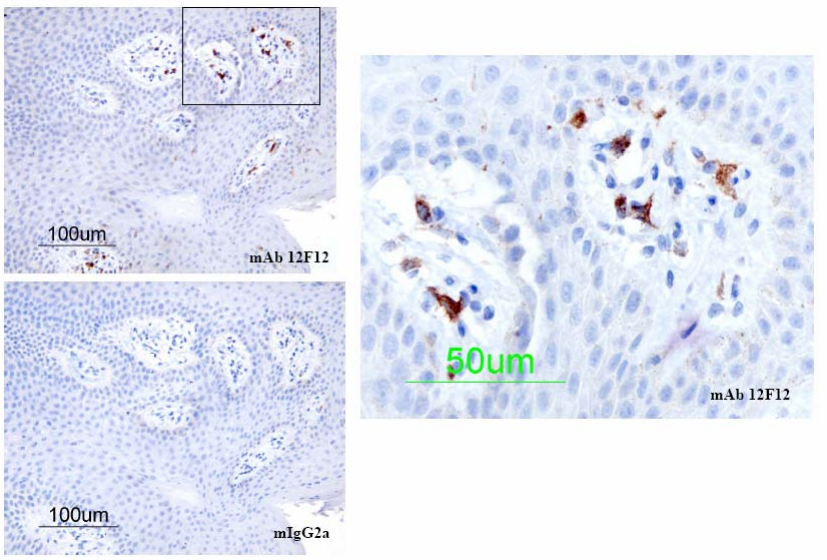

Figure 2.5 Presence of IL-23 in dendritic appearing cells. Expression of IL-23 protein (mAb 12F12) is demonstrated on dendritic appearing cells in the dermis of psoriasis. Isotype control staining is negative.

Interestingly, when immunohistochemical stainings of psoriatic sections were performed in our laboratories, the same Ab showed a diffuse positive pattern also in the epidermis, indicating that not only DCs, but also keratinocytes produced and secreted this cytokine (Figure 2.6). Similar staining pattern were already shown by Piskin and al. (Piskin, Sylva-Steenland et al. 2006). This group further analysed the biological activity of keratinocyte-produced IL-23 and demonstrated that the secreted cytokine was able to enhance IFN-γ expression by memory T cells. Therefore it is likely that KCs release IL-23, which in turn may affect infiltrating T cells, inducing the production of proinflammatory cytokines such as IL-17.

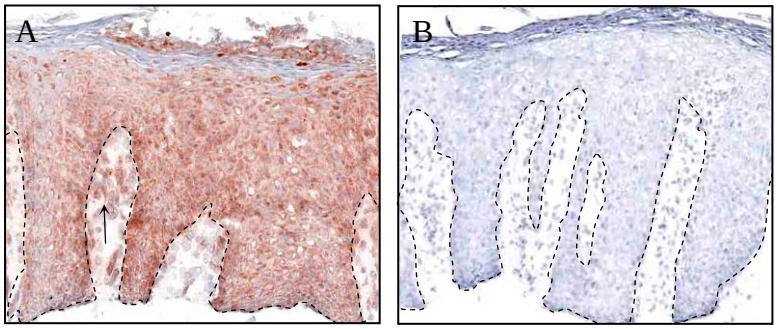

Figure 2.6 Immunohistochemical staining of IL-23. (A) Anti-IL23 showed an intense positive pattern in the epidermis. Arrow indicates IL-23 expression also in dermal cells. (B) Isotype control specimens.

2.2.2. In vivo evidence for a functional role of IL-23 in psoriasis

2.2.2.1. Injection of anti-IL-23 antibody inhibits development of psoriasis in the AGR psoriasis mouse model

The next step was to investigate whether the blockade of IL-23 signalling in vivo was sufficient to prevent the spontaneous conversion of uninvolved skin (PN) in psoriatic skin lesions (PP) in the AGR xenotransplantation psoriasis mouse model. Anti human-IL-23 neutralizing mAb, provided by Schering-Plough Biopharma, was injected subcutaneously at day 7 and day 21 and histological analyses of the grafts were performed at day 35. The effect of the anti-IL-23 mAb was compared to isotype control treatment and the current benchmark anti-TNF-α (Infliximab). Skin grafts from mice receiving isotype mAb developed a fully fledged psoriatic phenotype, showing an epidermal hyperplasia similar to the one observed in the lesional psoriatic skin of the donor patient (Figure 2.7B and D). In contrast, treatment with anti-IL-23 significantly inhibited the development of psoriasis, with grafts presenting a histological picture nearly indistinguishable from the uninvolved skin before transplantation. (Figure 2.7A and C). Acanthosis and papillomatosis indices were quantified in skin grafts of three independent experiments with PN skin from three different patients. There was a statistically significant reduction of psoriasis acanthosis ($p=0.0005$) and papillomatosis indices ($p=2.3x10^{-6}$) in grafts of mice injected with anti-IL-23 compared to isotype control treated mice (Figure 2.7E and F). Moreover anti-human IL-23 antibody demonstrated to have a similar inhibitory effect on psoriasis development as the well established anti-psoriatic drug anti-TNF-α (Infliximab).

The described results clearly indicate that IL-23 is required for the development of lesional psoriatic skin and that the neutralization of this cytokine can significantly prevent the onset of a psoriasiform chronic inflammatory reaction.

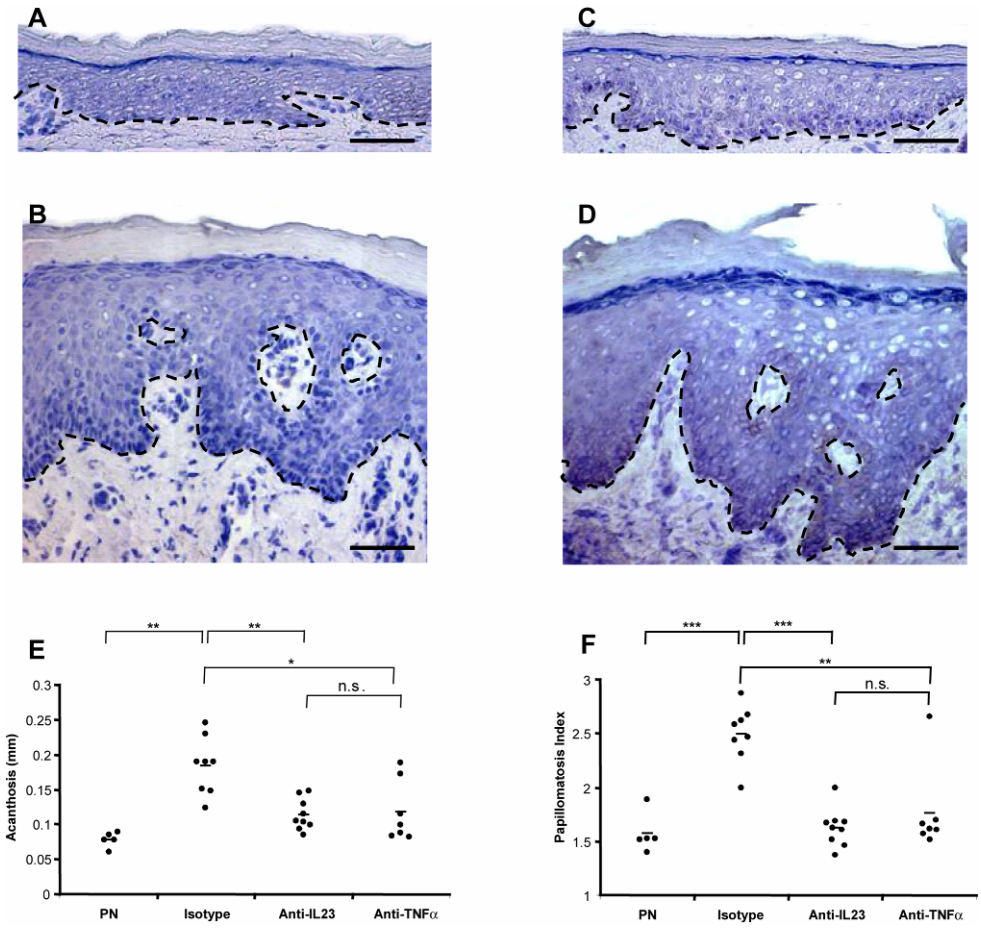

Figure 2.7 Blockade of IL-23 prevents development of psoriasis in a psoriasis xenotransplantation mouse model. Microscopic view of uninvolved psoriatic (PN) skin on the day of transplantation (A) and lesional psoriatic skin of the same patient (B). Uninvolved skin 5 weeks after transplantation onto AGR129 mice treated with anti-human IL-23 antibody (C) or isotype control antibody (D). Acanthosis (E) and papillomatosis indices (F) in skin grafts before transplantation onto AGR129 mice and after 35 days treatment with either anti-human IL-23 or isotype-matched control antibody. There was a statistically significant reduction of psoriasis acanthosis and papillomatosis indices in grafts of mice treated with anti-human IL-23 compared to isotype control mice. The efficacy of the treatment with anti-human IL-23 in inhibiting psoriasis development was comparable to anti-TNF-□infliximab), the „gold standard“ in psoriasis treatment. (F). Data in panel E and F were pooled together from three independent experiments with PN skin from three different patients. Dots represent independently grafted mice samples. Scale bars in A-D represent 20μm. P-values were calculated using the unpaired Student‘s t test. * p≤ 0.01,** p≤ 0.001, *** p≤0.0001

2.2.2.2. Anti-IL-23 therapy inhibits inflammatory T cell expansion

There is a strict relationship between the presence of T cells in a pre-psoriatic skin and the development of a psoriatic lesion. In fact previous work showed that the

blockade of T cell function after transplantation of PN skin onto AGR mice inhibited the development of a psoriatic lesion (Boyman, Hefti et al. 2004; Boyman, Conrad et al. 2006). Hence, the capacity of an antibody to block the proliferation of T cells reflects the efficacy of the treatment. In order to asses the impact of IL-23 blockade in this context, the number of CD3+ T in the engrafted skin on the day of transplantation and after 35 days were analysed. Transplants of mice treated with anti-IL23 mAb showed a significant reduction ($p< 0.001$) of total T cells number compared to grafts injected with isotype control mAb (Figure 2.8A). Moreover by analysing the distribution of these lymphocytes, isotype control treated mice showed an increase of the T cell epidermal to dermal ratio, whereby both anti-IL23 and anti-TNF-α treatment led to a reduction of this ratio (Figure 2.8B). Injection of anti-IL23 mAb therefore blocked the proliferation and the migration of T cells into the epidermis with an efficacy comparable to anti-TNF-α treatment, the "gold standard" in anti-psoriatic therapy.

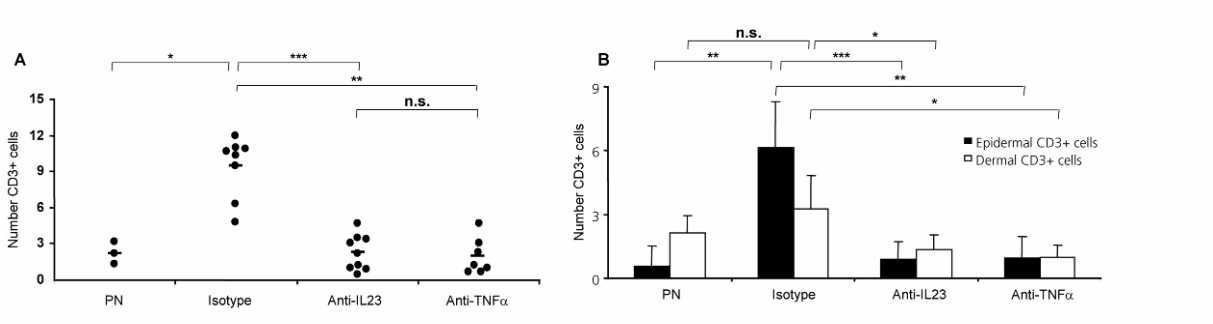

Figure 2.8 IL-23 blockade reduces the number of inflammatory T cells (A) Total human CD3+ T cell count in PN skin grafts before transplantation and 35 days after transplantation and treatment with either anti-human IL-23, isotype-matched control or anti-TNF-α (infliximab) antibody. There was a statistically significant reduction of CD3+ total T cells in grafts treated with anti-human IL-23 compared to isotype control treated mice. (B) Number of CD3+ T cells present in the epidermal and the dermal compartment. Treatment with anti-human IL-23 led to a significant reduction of CD3+ T cells in both the epidermis and dermis of grafted skin. Dots in panels A represent independently grafted mice samples and are representative of three independent experiments with PN skin from three different patients. P-values were calculated using the unpaired Student's t test.
* $p \leq 0.003$, ** $p \leq 7x10^{-4}$, *** $p < 7x10^{-6}$.

2.2.2.3. Increase in IL-23p19 mRNAs parallels the typical psoriatic changes in the epidermis

The advantage of the xenotransplantation mouse model used throughout this thesis is that uninvolved psoriatic skin engrafted on these mice spontaneously converts into a typical psoriatic plaque lesion within 35 days. This allows to

investigate all the immunological and phenotypical changes that occur during the conversion of uninvolved prepsoriatic skin (PN) into a fully-fledged psoriatic lesion (PP). In a recent work we showed that during the formation of a psoriatic lesion there is an increase and redistribution of CD3+ T cells in graft transplanted on the AGR mouse model (Conrad, Boyman et al. 2007). Specifically, during the first 2 weeks only a proliferation of dermal T cells was observed in the grafts in the absence of visible changes in the epidermis. Afterwards, around day 21, the migration and proliferation of T cells in the epidermal compartment induced the typical psoriasiform changes such as an increase in the papillomatosis and acanthosis indices. Based on the results of the previous paragraphs, which showed the importance of IL-23 during the formation of a psoriatic lesion, the next step was to investigate IL-23 production in relation to the development of a psoriatic plaque. Prepsoriatic skin of two different patients was engrafted on the AGR mice and analysed on day 0 and after 7, 21 and 35 days upon transplantation. RNA was extracted from grafts and quantitative RT-PCR was performed using human specific primers for IL-23p19. This experiment showed an increase of IL-23p19 mRNA levels during the course of the disease reaching a peak at day 35. By correlating the kinetics of mRNA expression for this cytokine with the proliferation of pathogenic T cells, a slight delay in IL-23p19 expression levels was seen compared to the expansion of CD3+ T cells (Figure 2.9A, C). This observation is in agreement with previous work which showed that IL-23 is not necessary for the proliferation of T cells, but is important for the induction and survival of the recently discovered Th-17 subpopulation, which have been associated with different autoimmune diseases, including psoriasis (Zheng, Danilenko et al. 2006). Moreover, IL-23p19 mRNA levels approximately paralleled the typical epidermal hyperplasia seen during the development of a psoriatic lesion (Figure 2.9B, D). A result that supports the observation of Zheng at al, who showed that IL-23 promotes the thickening of the epidermis through the induction of IL-22 and activation of Stat3 in KCs, a pathway critical for psoriasis development (Zheng, Danilenko et al. 2006).

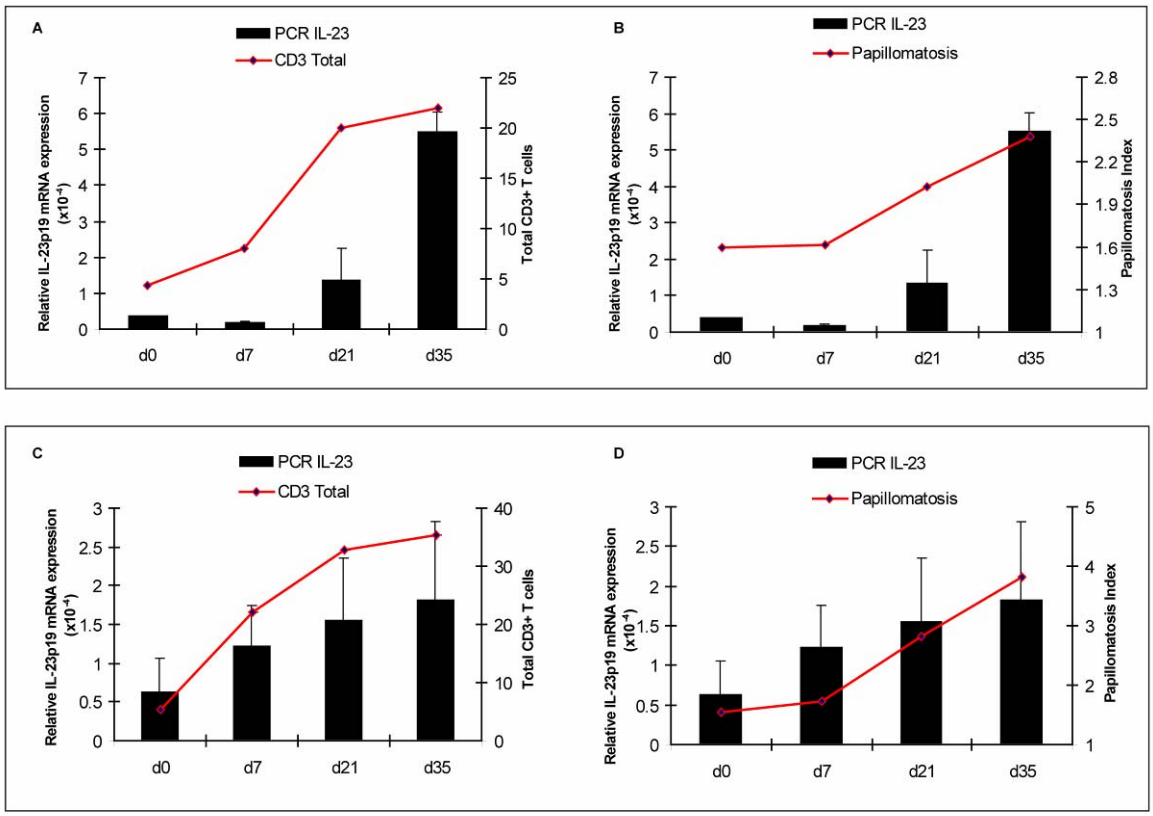

Figure 2.9 IL-23p19 mRNA expression during psoriasis development in the AGR-/- xenograft mouse model. Kinetics of human IL-23p19 mRNA expression (normalized to human GAPDH) during psoriasis development was compared to the number of total CD3+ T cells (A, C) and papillomatosis indices (B, D). Grafts were analysed on the day of transplantation and after 7, 21 and 35 days. RNA was extracted from grafts and quantitative RT-PCR was performed using human specific primers for IL-23p19. Experiments were repeated twice, with A, B representing one patient and C,D the second patient.

2.3. Discussion

IL-23 is a heterodimeric cytokine, belonging to the IL-12 family, and is mainly secreted by DCs and macrophages (Trinchieri, Pflanz et al. 2003). In the last years several studies have demonstrated the essential role of IL-23 in the regulation of cell mediated immune responses, with at least one of its activities being to support the survival of Th-17 cells. In different models of autoimmune disease, such as IBD, CIA and EAE, it was shown that the neutralization of IL-23 function significantly ameliorated disease pathogenesis (Kullberg, Jankovic et al. 2006)(Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003; Langrish, Chen et al. 2005). More recently, IL-23 was also found to be an essential mediator in skin inflammation. In fact, the direct injection of IL-23 in mouse skin was shown to induce psoriasisform changes like

dermal inflammation, erythema, and epidermal hyperplasia (Chan, Blumenschein et al. 2006)(Zheng, Danilenko et al. 2006). Finally, there are at least three different direct evidences for the involvement of IL-23 in the pathogenesis of psoriasis. First, psoriatic lesions have an increased expression of IL-23 mRNA and protein compared to uninvolved psoriatic skin and the skin of healthy donors (Lee, Trepicchio et al. 2004)(Piskin, Sylva-Steenland et al. 2006)(Chan, Blumenschein et al. 2006). Secondly, a humanized mAb targeting the p40 subunit of both IL-12 and IL-23 already reached the phase II of a clinical trial, demonstrating the efficacy of inhibiting IL-23 function in psoriasis (Kauffman, 2004; Krueger, 2007}. Finally, two single nucleotide polymorphisms in the IL-23R gene locus have been reported to highly associate with psoriasis (Capon, Di Meglio et al. 2007; Cargill, Schrodi et al. 2007). Although all these studies suggest an imporatance of the IL-23 pathway in psoriasis, no data are available regarding the specific role and function of this cytokine during disease development.

The first part of this study focused on the expression of IL-23R in tissues of patients with psoriasis. The analyses were done on cells freshly isolated from the peripheral blood and stained with a specific mAb to the IL-23R chain. Flow cytometry analyses demonstrated a significant increase in IL-23R expression on CD3+ and CD4+ cells of patients with psoriasis (n=12) compared to healthy donors (n=13). Approximately 30% and 10% of peripheral monocytes/DCs and B cells respectively stained positive for IL-23R, with psoriatic patients having a slight increases in the levels of expression on both populations.

As psoriasis is mainly a skin disease the next step was to analyse cells isolated from the epidermal and dermal compartment of psoriatic lesions (n=3). Dermal DCs and T cells showed the highest levels of IL-23R with a significant increase compared to the same cells residing in the skin of healthy individuals. In contrast, no difference in the pattern of expression was detected among psoriatic and healthy KCs. Collectively, these data indicate that in psoriasis there is a dysregulation of IL-23R expression, which may lead to the aberrant activation of immune cells and induce a pro-inflammatory cascade leading to the typical psoriasiform changes.

One typical feature of psoriatic lesions is the large increase of mature DCs compared to the immature state of these cells in the symptomless psoriatic skin (Nestle, Turka et al. 1994; Lee, Trepicchio et al. 2004). During this study

immunohistochemical staining of psoriatic skin section showed expression of IL-23 on dendritic appearing cells in the dermis, a result that agrees with other studies where human mature DCs were shown to secrete IL-23 in response to different TLR agonists (Vanden Eijnden, Goriely et al. 2006). Taken together these two results indicated that IL-23 may be produced by dermal DCs as a consequence of a still undefined external trigger. The fact that an excessive production of IL-23 can lead to an overreaction of the immune response has been shown in different mouse models. For example, mice constitutively expressing the p40 subunit of IL-12/IL-23 in the basal layer of the epidermis had an enhanced production of IL-23, but not IL-12, and spontaneously developed an inflammatory skin disease with increased numbers of T cells, neutrophilis and macrophages (Kopp, Lenz et al. 2003). Moreover, transgenic mice expressing mouse IL23p19 mRNA in multiple tissues showed systemic inflammation, impaired growth, and premature death (Wiekowski, Leach et al. 2001).

In order to study the in-vivo relevance of IL-23 in psoriasis, keratomes from uninvolved psoriatic skin were xenografted in an established mouse model of psoriasis (Boyman, Hefti et al. 2004; Nestle, Conrad et al. 2005; Conrad, Boyman et al. 2007). Previous studies have shown that after engraftment, transplants developed a typical psoriatic lesion over the course of 5 weeks, characterised by epidermal hyperplasia (acanthosis,) elongation of the rete ridges (papillomatosis) and increased number of dermal and epidermal T cells. During this study transplanted mice were treated with either neutralizing mAb to IL-23 or isotype matched control Ab and grafts were then analysed after 35 days. Immunohistochemestry evaluation of acanthosis and papillomatosis indices demonstrated that IL-23 treatment had the same efficacy in inhibiting psoriasis development, as TNF-α, the “gold standard” in anti-psoriatic therapy. Mice treated with isotype mAb instead developed into a full-fledged psoriatic lesion, with epidermal hyperplasia reaching similar levels as the lesional psoriatic skin of the donor patient. IL-23 neutralization significantly reduced also the activation and expansion of pathogenic T cells, thereby confirming the importance that this cytokine has on the immune components and on the skin environment as well.

Our results are in agreement with previous finding in the EAE animal model, which showed that IL-23 is important during the development and recruitment of inflammatory cells to the CNS (Thakker, Leach et al. 2007). A similar scenario can therefore be postulated in psoriasis. It is likely, that IL-23 is important during the

development of the psoriatic plaque as it triggers a proinflammatory cytokine cascade responsible for epidermal hyperplasia and the survival of Th-17 cells. In line with these findings, IL-23 mRNA levels were shown to slowly increase during the development of a psoriatic lesion, reaching a peak five weeks after transplantation. The kinetics of this cytokine paralleled the development of epidermal hyperplasia demonstrating the important role of this cytokine in relation to psoriatic epidermal hyperplasia. IL-23 expression levels coincided or slightly followed the increase of CD3+ T cells in the transplanted skin, suggesting that IL-23 could have a role in supporting the survival of lesional Th-17 cells and thereby also their pathogenic function, primly mediated by the release of IL-17 and IL-22 (Acosta-Rodriguez, Rivino et al. 2007)(Zheng, Danilenko et al. 2006)(Liang, Tan et al. 2006).

All these data together point to a fundamental role of IL-23 during the pathogenesis of psoriasis. Moreover the in vivo data presented in this chapter, together with the encouraging results obtained in initial clinical trials with anti-IL-12p40 in psoriasis suggest that IL-23 may be a good candidate for the treatment of psoriasis. However, it will be important to determine the long-term effects of IL-23 neutralization in terms of efficacy and safety.

Chapter 3

Role of IL-17 in psoriasis

3. Role of IL-17 in psoriasis

3.1. Introduction

The putative role of IL-23 in the induction of autoimmune disease has already been discussed. However, the exact mechanism by which IL-23 may cause the development of chronic inflammatory diseases is still unclear. Several recent studies suggested that T helper (h)-17 T cell subpopulation, whose survival and expansion seems to depend on IL-23, may account for the generation and/or maintenance of autoimmune disorders.

Th-17 cells are a type of Th cells committed to produce IL-17. This cytokine, originally called CTLA-8 (cytotoxic T lymphocyte-associated 8) was isolated from a murine CTL hybridoma cDNA library (Rouvier, Luciani et al. 1993), and subsequently renamed IL-17. Five homologous cytokines were later identified through database homology searches and were called IL-17 B-E (Weaver, Hatton et al. 2007). These cytokines, together with IL-17 (sometimes also called IL-17A) form the IL-17 cytokine family. Mouse and human IL-17 members display significant homology as shown in Table 3.1.

Table 3.1 Human IL-17 family members.

Family member	Alternative names	Chromosomal location	Homology of murine to human (%)	Cellular sources	Receptor
IL-17A	CTLA-8	6p12	62	Memory T cells	IL-17R
IL-17B	IL-20	5q32-34	88	pancreas, small intestine, stomach	IL-17RB
IL-17C	Cx2	16q24	75		unknown
IL-17D	IL-27	13q12.11	82	pancreas, small intestine, stomach	IL-17R
IL-17E	IL-25	14q11.2	76	several tissues(brain, kidney, lung, prostate, testis)	IL-17RB
IL-17F	ML-1/IL-24	6p12	54	Activated CD4 cells	IL-17R

IL-17 was initially described as a cytokine produced by activated/memory CD4+ T cells. However, further studies demonstrated that also CD8+ memory T cells, $\gamma\delta$ T cells, NK cells and neutrophils secrete this protein (Weaver, Hatton et al. 2007). The human receptor for IL-17 has a broad distribution, suggesting that IL-17 has the

capacity to induce different biological activities depending on which type of cells it is acting on (Afzali, Lombardi et al. 2007). In particular, IL-17 induces macrophages, fibroblasts, endothelial and epithelial cells to secrete proinflammatory cytokines and prostaglandins, which in turn mediate chemotaxis of neutrophils and monocytes to the sites of inflammation. Finally, this cytokine acts as a bridge between innate and adaptive immunity inducing the expression of the co-stimulatory molecule ICAM-1, which supports T cell activation (Afzali, Lombardi et al. 2007).

At the same time when Cua et al. reported that IL-23 and not IL-12 was crucial for autoimmune inflammation in the brain (Cua, Sherlock et al. 2003), the group of Aggarwal et al demonstrated that IL-23 induces the production of IL-17 from memory, and not naïve, T cells (Aggarwal, Ghilardi et al. 2003). Further studies demonstrated that these IL-17 producing cells were responsible for the development of autoimmune inflammation (Langrish, Chen et al. 2005). It was shown that Th-17 cells primed for reactivity to the central nervous system and cultured in vitro with recombinant IL-23, induced EAE once transferred into recipient mice. In contrast, primed Th1 cells expanded with IL-12 were not pathogenic. In a subsequent step, the gene expression profile of IL-12-driven Th1 and IL-23-driven Th-17 cells were analysed. Whereas Th1 cells were preferentially expressing genes associated with cytotoxicity and host defence, such as IFN-γ, granzymes and FasL, the IL-23 stimulated cells were mainly producing IL-17, IL-17F, TNF-α and IL-6 which are expressed during organ-specific inflammation, but not during immune surveillance (Langrish, Chen et al. 2005). Finally, to prove the direct contribution of IL-17 and not of IFN-γ to EAE, mice were treated with either anti-IFN-γ or anti-IL-17 mAbs. The neutralization of IFN-γ led to disease exacerbation due to the high levels of IL-17 in the serum. Whereas anti-IL-17 treatment partially protected the mice from EAE, confirming previous works which showed that IL-17 deficient mice were resistant to induced EAE and CIA (Nakae, Nambu et al. 2003). In the following months, further studies, confirming the reciprocal interaction between IFN-γ and IL-17 were published (Harrington, Hatton et al. 2005; Park, Li et al. 2005). These studies showed that Th1 and Th2 cytokines strongly inhibited the generation of Th-17 cells, whereas the neutralization of IFN-γ and IL-4 were increasing IL-17 production. Additionally, IFN-γ knock-out mice had enhanced IL-17 production caused by loss of IFN-γ suppression (Chu, Swart et al. 2007). All these data together supported the hypothesis that IL-17 producing cells might

develop via a lineage distinct form Th1 and Th2. In fact, this hypothesis was soon confirmed by three independent groups (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006), which showed that, in mice, TGF-β and IL-6 were required for the development of IL-17 secreting cells. It was demonstrated that TGF-β alone induced the production of Treg cells, while the synergy of TGF-β and IL-6 promoted the differentiation of Th-17 cells. Thus, it is current belief that naïve T cells will differentiate to Th-17 cells in the presence of TGF-β and IL-6 and that IL-23 will maintain and/or expand already differentiate Th-17 cells. However, the exact mechanism by which IL-23 promotes Th-17 response remains to be established.

IL-17 producing cells have been extensively studied in mice but much less is known about their identity and properties in humans. The group of Lanzavecchia (Acosta-Rodriguez, Rivino et al. 2007) provided the first detailed characterisation of human Th-17 cells. They distinguished human memory Th-17 cells from Th1 and Th2 on the basis of selective expression of chemokine receptors. In particular, Th-17 cells co-expressed CCR6, a mucosa-homing receptor, and CCR4, a skin-homing receptor. In contrast CCR6+/CXCR3+ cells identified a heterogeneous population mainly composed of Th1 cells and to a lesser extent of T cells producing both IL-17 and IFN-γ. They also investigated the signals driving human Th-17 differentiation and found that TGF-β alone or together with IL-6 failed to induce differentiation of IL-17 cells. IL-1β induced the differentiation of IL-17 producing cells and synergised with IL-6 to promote Th-17 development. Accordingly, IL-1β up-regulated the transcription factor RORγT (retinocic-acid-receptor-related orphan receptor-γT) which is known to be required for the differentiation of mouse Th-17. Recently these results were confirmed and extended by several other groups (Acosta-Rodriguez, Napolitani et al. 2007; Annunziato, Cosmi et al. 2007; Wilson, Boniface et al. 2007; Yang, Panopoulos et al. 2007). In particular Annunziato and co-workers provided the first detailed characterisation of human Th-17 cells isolated from the gut of patients with Crohn's disease. They demonstrated the existence of both Th-17 and IFN-γ-producing Th-17 cells in the gut mucosa and identified IL-23R, CCR6 and RORγT as Th-17 specific markers (Annunziato, Cosmi et al. 2007).

Th-17 cells play an important role in sustaining autoimmune disorders, such as EAE and CIA. Moreover IL-17 has been implicated in a variety of other chronic human inflammatory diseases. Increased levels of this cytokine have been demonstrated in respiratory diseases, ulcerative colitis, Crohn's disease, multiple sclerosis and psoriasis (Steinman 2007). During skin inflammation IL-17 may act alone or together wit IFN-γ and/or TNF-α to enhance the production of IL-6, IL-8, GM-CSF and adhesion molecules on keratinocytes which in turn promotes the chemotaxis of leukocytes (Teunissen, Koomen et al. 1998; Albanesi, Cavani et al. 1999).

In this chapter the regulation and the function of IL-17 producing cells *in vitro* and *in vivo* was studied. In the first part of this work Th-17 cells were analysed in the blood and in the skin of psoriatic patients and compared to healthy volunteers. Blood PBMCs were analysed by flow cytometry in order to investigate the cytokine profile of lymphocytes. In particular, IFN-γ and IL-17 production by CD3+ T cells were investigated. Considering that psoriasis is mainly a skin disease and that all the major phenotypical and morphological changes take place in this tissue, the same flow cytometry analyses were performed in cells isolated from the skin. Finally, to confirm the results obtained in the previous experiments, the mRNA expression of IL-17 and IFN-γ were also analysed by real time polymerase chain reaction (RT-PCR). All these experiments show an enhanced expression of IL-17 in both blood and skin of psoriatic patients relative to healthy controls and suggest a dysregulation in the production and probably function of this cytokine in psoriasis. In order to analyse the functional role of IL-17 during the development of a psoriatic lesion, AGR mice were transplanted with uninvolved psoriatic skin and treated with a neutralizing antibody against IL-17. Although anti-IL-17 treatment did not ameliorate psoriasiform lesion formation in a significant manner, the increased production of IL-17 in the blood and especially in the skin of psoriatic patients lends support to the argument that Th-17 cells may play an important role in psoriasis.

3.2. Results

3.2.1. Th-17 cells in psoriasis

3.2.1.1. Increased production of IL-17 by psoriatic T cells

In the previous chapter I have demonstrated the importance of the IL-23 pathway in psoriasis. DCs and T cells were both positive for the IL-23R and psoriatic patient had an enhanced level of expression in the blood and the skin, compared to healthy donors. Based on the evidence that IL-23 is required for the effector functions and the recruitment of Th-17 cells at the site of inflammation the next step was to analyse the effect of IL-23 on IL-17 production on activated CD3 T cells.

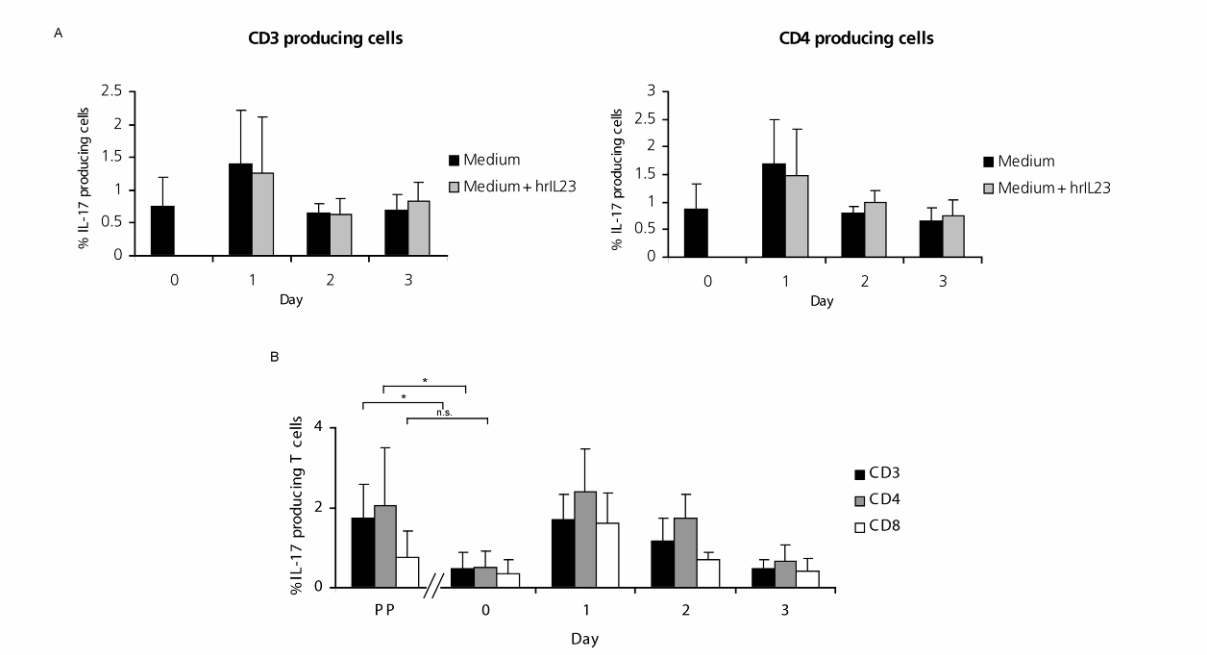

Figure 3.1 Increased production of IL-17 by psoriatic T cells (A) T cells freshly isolated from the blood of healthy donors (n=6) were treated with anti-CD3/CD28 monoclonal antibodies in the presence or absence of recombinant IL-23 (100ng/ml) over 3 days. Addition of IL-23 did not increase the secretion of IL-17 over the 3 days of analysis. (B) Psoriatic T cells showed increased production of IL-17 as compared to T cells of healthy donors. CD3+ T cells were isolated from the blood of healthy donors and treated with anti-CD3/CD28 monoclonal antibodies in the presence of recombinant IL-23 (100ng/ml) over 3 days. Immediately after isolation, T cells from blood of psoriatic patient (PP) produced similar levels of IL-17 as normal T cells after 1 day of stimulation. Graph represent mean of eleven or six experiments performed on different psoriatic patients and healthy donors respectively.
* p=0.006, ** p=0.015

In a first step T cells from the peripheral blood of healthy donors were isolated by magnetic depletion. These cells were then stimulated over 3 days with anti-CD3 and anti-CD28 mAbs in the presence or absence of human recombinant IL-23 (hrIL-23)

(100 ng/ml). By using flow cytometry, the levels of IL-17 production were analysed on the day of extraction and after stimulation. Freshly isolated T cells already showed production of IL-17 and this secretion was enhanced after 1 day of stimulation and progressively decreased over the 3 days of analysis. Interestingly, under these culture conditions the rates of IL-17 production were not altered by hrIL-23 (Figure 3.1A). Based on several studies published in the last years, this result is not surprising. In fact, it has been demonstrated that IL-23 do no effect the production of IL-17, but may rather function in the maintenance of Th-17 committed cells (Aggarwal, Ghilardi et al. 2003; Suryani and Sutton 2007). When analysing freshly isolated psoriatic T cells (n=11), significantly higher levels of this cytokine were detected compared to T cells isolated from healthy donors (n=6). IL-17 production of freshly isolated psoriatic T cells was comparable to IL-17 production of normal T cells after 1 day of stimulation with hrIL-23 and anti-CD3/CD28 (Figure 3.1B). Among the CD3+ cells, the CD4+ subpopulation had the highest levels of IL-17 production.

3.2.1.2. Cytokine profile of activated T cells in the blood and in the tissue

To investigate if the previously identified Th-17 cells were able to produce Th1 type cytokines, flow cytometry analyses were done on peripheral T cells. The cytokine profile of CD3+ T cells from the blood of psoriatic patients (n=9) were compared to that of healthy individuals (n=8). The majority of the cells secreted either IFN-γ or IL-17, with only few cells producing both cytokines at the same time (Figure 3.2C). T cells from psoriatic patients produced higher levels of IFN-γ and IL-17 compared to healthy donors (Figure 3.2A). Especially psoriatic CD4+ T cells had a higher percentage of IL-17 producing T cells compared to healthy donors (Figure 3.2B). This experiment demonstrates that the majority of T cells contain either IFN-γ or IL-17, suggesting a dichotomy of IFN-γ and IL-17 producing T cells in the blood of psoriatic patients and healthy individuals.

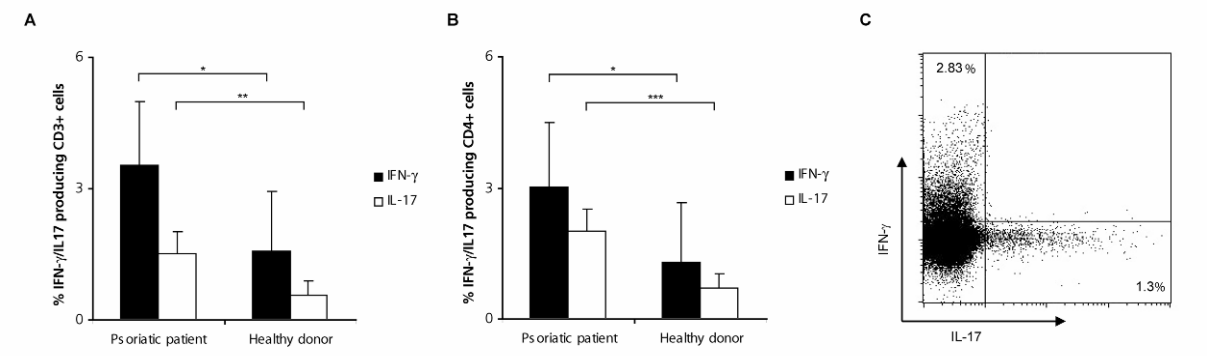

Figure 3.2 Cytokine profile of peripheral blood T cells. (A-B) CD3+ and CD4+ T cells freshly isolated from peripheral blood of psoriatic patient had an increased production of IL-17 and IFN-γ when compared to healthy donor cells. (C) Representative flow cytometry analysis of IL-17 and IFN-γ production by CD3+ T cells present in the peripheral blood (n=9 for psoriatic patient, n=8 for healthy volunteers). Plots are gated on lymphocytes as identified by using forward and side scatter settings. *p≤ 0.005, **p=0.0001, ***p=2x10^{-5}

The following step was to analyse the secretion levels of these two cytokines in skin lymphocytes. When CD3+ T cells were freshly isolated from the skin of psoriatic patients (n=7), epidermal CD3+ T cells produced significantly higher amounts of both IFN-γ and IL-17 compared to lymphocytes extracted from the dermis of a psoriatic lesion and of healthy skin (n=3) (Figure 3.3A). CD3+ T cells present in the dermis of psoriatic lesions were secreting higher amounts of IFN-γ compared to the lymphocytes residing in the skin of healthy individuals, albeit not reaching statistical significance. Interestingly, in the epidermal compartment, in addition to the either IFN-γ or IL-17 producing cells a consistent major subpopulation secreting both of these two cytokines was identified. In contrast, in the dermis of psoriatic patients and healthy controls the amounts of "double producers" was very low. These results suggest that IL-17 producing cells preferentially migrate into the psoriatic epidermis, where they exert their pathogenic effect by secreting IL-17 (and in a significant proportion IFN-γ). This cytokine upregulates the expression of chemokines on epithelial tissues, thereby attracting further immune cells to the epidermis (Weaver, Hatton et al. 2007).

Taken together, these results show that psoriasis correlates with increased frequencies of IFN-γ and IL-17-producing cells in the skin, suggesting that both Th1 and Th-17 cells may contribute to the propagation of the disease. Support for this hypothesis comes from two studies performed on intestinal inflammation models (Hue, Ahern et al. 2006; Kullberg, Jankovic et al. 2006). Herein it was demonstrated that both Th1 and Th-17 cells were present during the development of colitis, which

let the authors arguing that the two cell responses may synergize to exacerbate disease pathology.

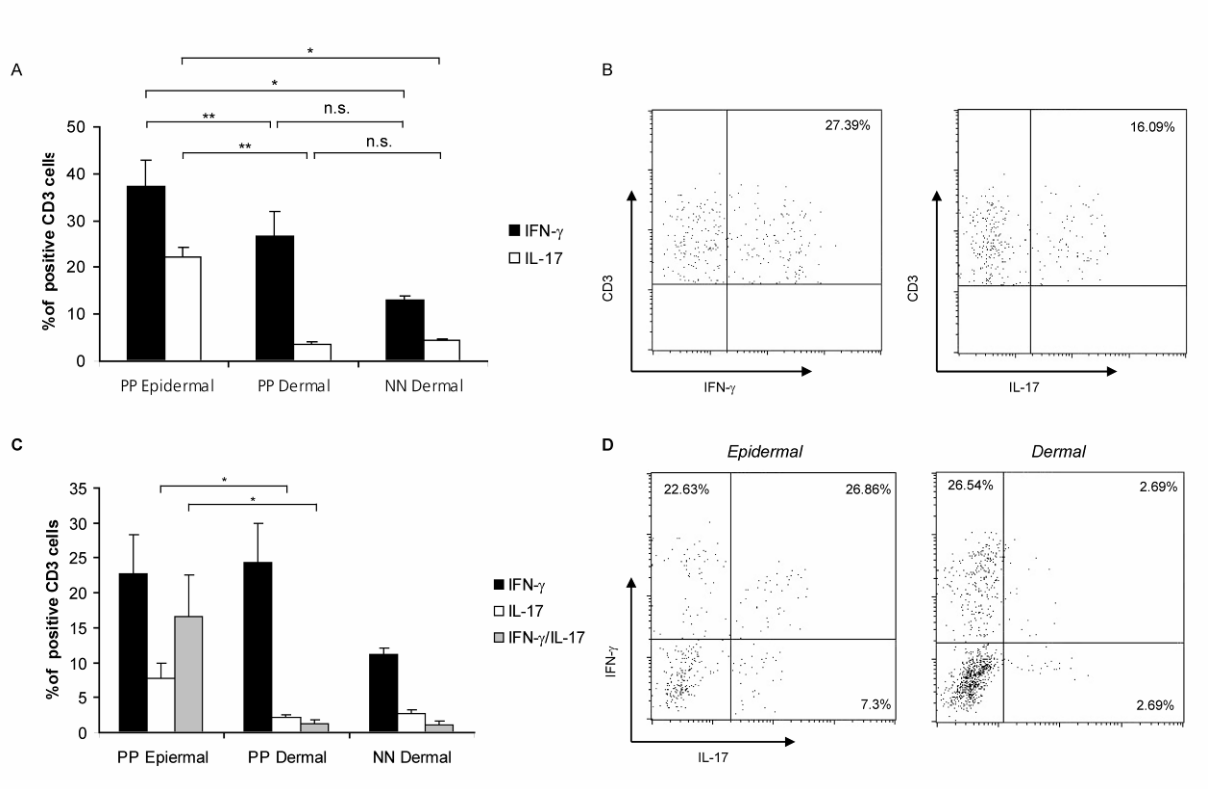

Figure 3.3 Cytokine profile of CD3+ cells present in the skin. Cells were freshly isolated from the epidermal and dermal compartment of psoriatic patients (PP, n=7) and healthy individuals (NN, n=3). (A) Percentage of CD3+ cells producing either IFN-γ or IL-17. (B) Representative flow cytometry analysis of total Th-17 and Th1 cells present in the epidermal compartment. (C) Better characterisation of the cytokine profile of CD3+ cells present in the skin of psoriatic patients and healthy individuals. (D) Representative flow cytometry analysis of IL-17 and IFN-γ production by CD3+ T cells present in the epidermal and dermal compartment of a psoriatic lesion. (B and D) Numbers in quadrant indicate percentage of positive cells as assessed by intracellular cytokine staining. Plots are gated on lymphocytes as identified by using forward and side scatter settings. *p≤ 0.03, **p≤0.008

3.2.1.3. Characterization of IL-17 producing T cells

To better characterise the Th-17 population, peripheral T cells were analysed according to their expression of surface markers for activated/memory T cells. Classically, T cells with different specificities are continually produced in the bone marrow in order to maintain an efficient T cell repertoire, capable of responding to any antigen. Lymphocytes that survive thymic selection are released in the periphery in a naïve state. After encountering the appropriate antigen, T cells undergo clonal expansion and differentiation into effector and memory T cells. Two types of memory T cells are present in the peripheral blood and are characterised by different

migratory capacities. “Central” memory T cells (T_{CM}) expressing the chemokine receptor CCR7 and representing a pool of cells that home to secondary lymphoid organs. Effector memory T cells (T_{EM}) do not express this receptor and predominantly migrate to peripheral tissues to mediate inflammatory reactions. Human naïve T cells in addition to the CCR7 receptor also express the human leukocyte antigen, CD45RA.

To investigate the phenotype of the Th-17 population, peripheral cells of healthy donors and psoriatic patients were stained with mAbs directed against IL-17, CD45RA and CCR7 and subsequently analysed using flow cytometry (Figure 3.4A - D). Within the IL-17–cell population, most cells were CCR7+CD45RA- or CCR7-CD45RA-, indicating that these cells had the phenotype that has been associated with central memory (CCR7+CD45RA-) or effector memory cells (CCR7-CD45RA-). The Th-17 population in the blood of healthy donors (n=7) and psoriatic patients (n=11) displayed similar expression of surface markers for activated/memory T cells.

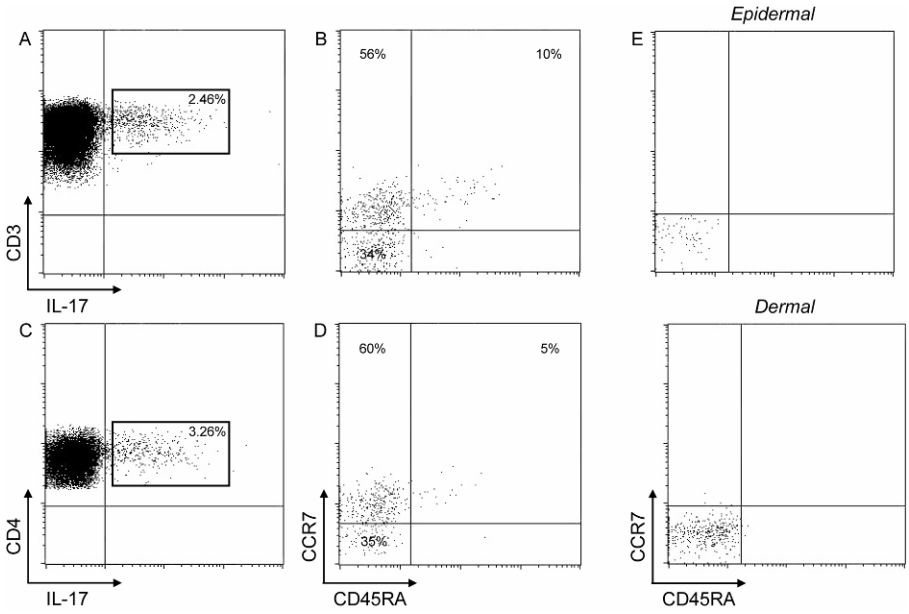

Figure 3.4 Characterization of IL-17 producing T cells. (A ,C) Representative example of IL-17 producing CD3+ and CD4+ T cells freshly isolated from peripheral blood of psoriatic patients (n=11). Numbers in quadrants represent percentage of cells staining positive for IL-17. To further define the Th-17 subpopulation, cells were analysed according to their CD45RA and CCR7 phenotype (B, D). IL-17 is mainly produced by memory T cells (CD45RA- CCR7-/+). (E) Representative example of surface markers for activated/memory T cells expressed by epidermal and dermal Th-17 cells extracted from the same lesional psoriatic skin. Cells were extracted from the epidermal and dermal compartment of psoriatic lesions (n=2) and subsequntially stained with mAb to IL-17, CD3, CD45RA and CCR7. Plots are gated on lymphocytes as identified by using forward and side scatter settings.

Subsequently, the expression of these surface markers was investigated in the Th-17 population found in the in the skin of psoriatic patients (n=2). Both IL-17 producing cells of the dermal and epidermal compartment show to be CCR7-/CD45RA-, indicating that they were all effector memory T cells as expected (Figure 3.4E).

3.2.2. Pro-inflammatory cytokines in human psoriatic skin

In order to analyse IL-17 and IFN-γ mRNA expression in lesional and non lesional psoriatic skin samples quantitative RT-PCR was performed. Both IL-17 and IFN-γ mRNA expression was increased in lesional psoriatic skin compared to non lesional skin from psoriatic patients (Figure 3.5). TNF-α, a validated target in many inflammatory diseases, was also expressed at significant higher levels in lesional psoriatic skin compared to non lesional skin from psoriatic patients. The relative amounts of TNF-α mRNA were at least a thousand times higher than the ones observed for IL-17 and IFN-γ, indicating the key role of this cytokine in the inflammatory process of psoriasis and explaining the efficacy of TNF-α blockers in the treatment of this skin disease.

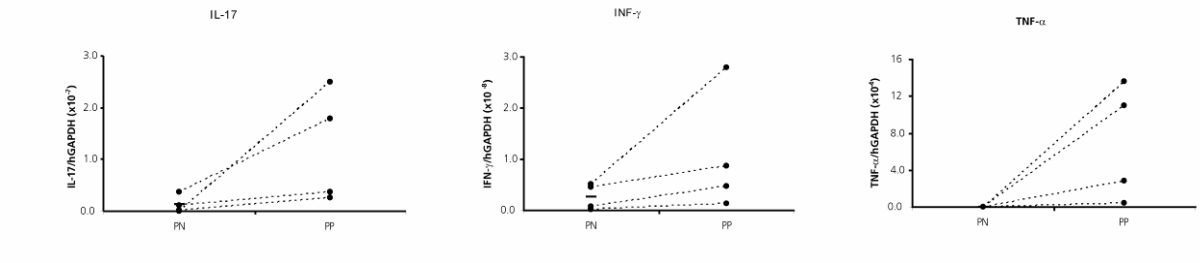

Figure 3.5 Quantification of pro-inflammatory cytokines expression in psoriatic skin. IL-17, IFN-γ and TNF-α mRNA expressions were increased in lesional psoriatic skin (PP) compared to non lesional skin (PN) from psoriatic patients. mRNA levels of each cytokine were normalised to their correspondent hGAPDH concentrations.

3.2.3. Blockade of IL-17 in vivo does not prevent the development of a psoriatic lesion

One of the final steps was to investigate if the high amounts of IL-17 at mRNA and protein level detected in the lesional psoriatic skin were responsible for the chronic inflammation seen in psoriasis. Injection of a human IL-17 specific mAb did not inhibit

the development of a psoriatic lesion in significant manner when using the AGR xenograft psoriasis model. For this experiment keratomes of non lesional psoriatic skin from 3 different patients were transplanted onto AGR mice and injected with anti-IL-17 mAb and matched isotype control mAb starting at day 7.

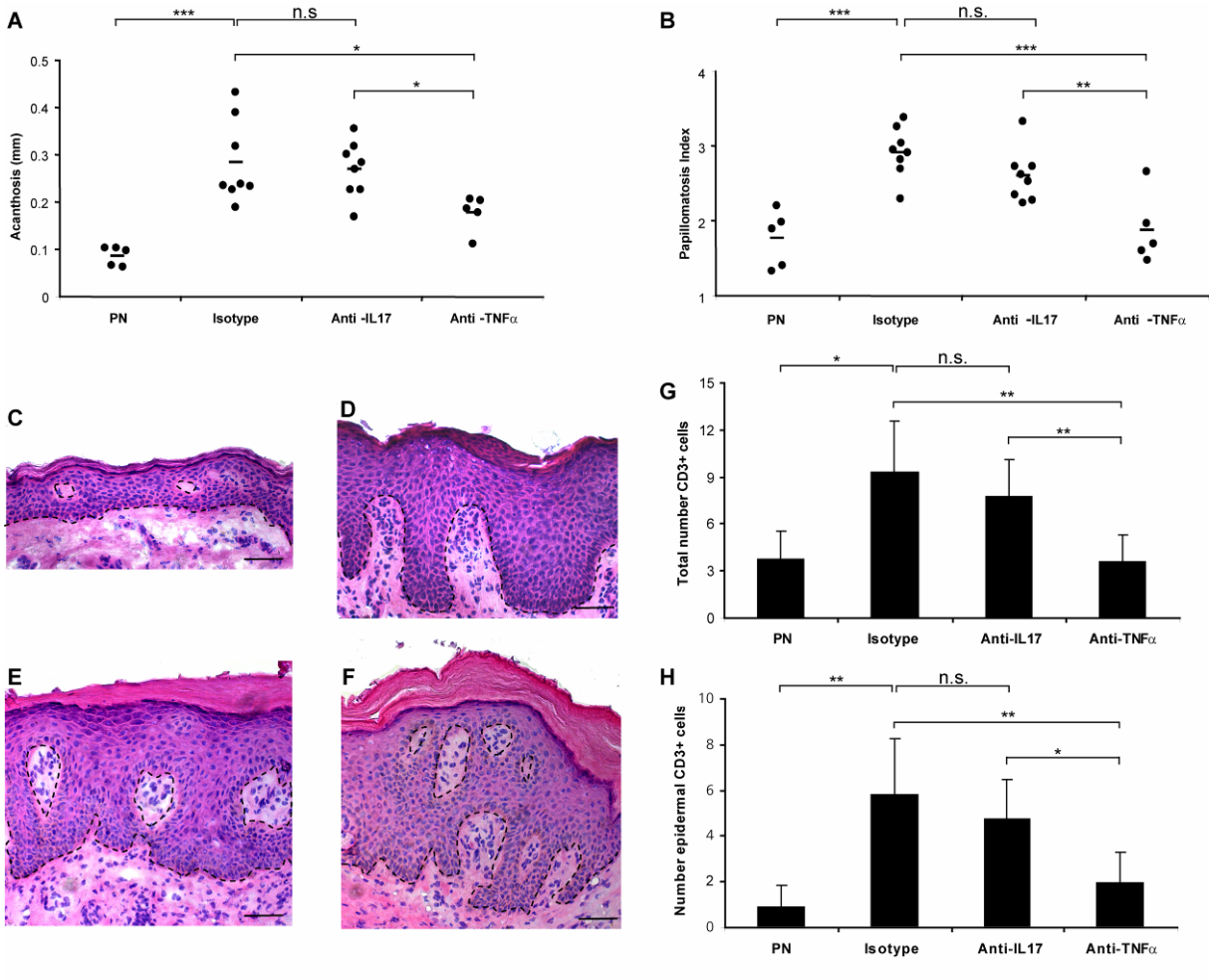

Figure 3.6 Neutralization of IL-17 function does not prevent the development of psoriasis. Acanthosis and papillomatosis indices in skin grafts before transplantation (PN) onto AGR129 mice and after 35 days treatment with either anti-human IL-17 or isotype-matched control antibody. There was no statistically significant reduction of psoriasis acanthosis (A) and papillomatosis indices (B) in grafts of mice treated with anti-human IL-17 compared to isotype control mice. Mice treated with anti-TNF-α (infliximab), had a statistically significant reduction of both acanthosis and papillomatosis compared to mice injected with anti-human IL-17 and isotype-matched control antibody. Microscopic view of uninvolved psoriatic (PN) skin on the day of transplantation (C) and lesional psoriatic skin (PP) of the same patient (D). Uninvolved psoriatic skin 5 weeks after transplantation onto AGR mice and after treatment with either anti-IL17 (E) or isotype-matched control mAb (F). (A-B) Data were pooled together from three independent experiments with PN skin from three different patients. Dots represent independently grafted mice samples. Scale bars in C-F represent 20 μm. P-values were calculated using the unpaired Student's t test. *p≤ 0.03, **p≤ 0.009, *** p≤ 0.0007

Anti-TNF-α (infliximab) was administered as positive control. At day 0 and 35 days after transplantation grafts were evaluated for their psoriatic phenotype, defined by the typical epidermal hyperplasia involving thickening of the epidermis (acanthosis) and the elongation of epidermal papillae (papillomatosis index) (Figure 3.6 A-B). The psoriatic phenotype was confirmed by immunohistochemestry (Figure 3.6 C-F). Mice treated with anti-IL-17 mAb and isotype control group presented similar values of both acanthosis and papillomatosis indices. Treatment with anti-TNF-α led to a statistically significant reduction of epidermal hyperplasia when compared to anti-IL17 or isotype control intervention. Numbers of total T cells and epidermal T cells were present in the human grafts treated with anti-IL17 or isotype control mAbs 35 days after engraftment onto AGR mice (Figure 3.6 G-H). These results suggest that IL-17 blockade alone is not sufficient to inhibit psoriasis development.

3.3. Discussion

The present study provides evidence that although Th-17 cells take part in the inflammatory reaction during the formation of a psoriatic lesion, the inhibition of IL-17 function is not sufficient to significantly block disease onset. Interestingly, in some models of inflammatory diseases, such as EAE and CIA, the neutralization of IL-17 function was efficacious in inhibiting the disease manifestation. For example, in the EAE model anti-IL-17 mAbs was shown to reduce the incidence and the severity of disease, even though cells extracted from the lymph nodes and the spleen retained their capacity to produce pro-inflammatory cytokines (IL-17, TNF-α, IL-2 and IFN-γ). However, these cells were not able to induce any pathogenic effect as only few lymphocytes and macrophages were found in the CNS, where the disease takes place. Blockade of IL-17 inhibited the upregulation of chemokines, which are important for T cell and macrophages migration (Park, Li et al. 2005).

The exact role of the Th-17 population in psoriasis is still unknown. The results presented in this chapter clearly show that peripheral T cells of psoriatic patients secrete higher levels of IL-17 compared to healthy control volunteers, and that the major producer of this cytokine was a subset of memory CD4+ T cells. Furthermore, the incubation of CD3+ T cells with anti-CD3 and anti-CD28 enhanced the secretion

of IL-17 with maximum levels reached after 1 day of stimulation. Since psoriasis is a skin inflammatory disease, where T cells have been reported to be the main effector cells promoting disease onset (Boyman, Hefti et al. 2004; Nickoloff and Nestle 2004; Boyman, Conrad et al. 2006; Conrad, Boyman et al. 2007), IL-17 and IFN-γ production in CD3+ skin T cells population was further investigated. These analyses showed a functional heterogeneity, in that a major population produced IFN-γ but not IL-17, a minor subset only IL-17 and a third population both cytokines together. This scenario differs from that seen in the blood where IFN-γ and IL-17 production was almost mutually exclusive. The three above mentioned subpopulations were particularly evident in the epidermal compartment of psoriatic lesions, whereas in the dermis IFN-γ- producing cells were the main subset. The Th1 cells were also the main population present in the dermal compartment of healthy individuals, however containing less IFN-γ producing cells then the dermis of psoriatic patients. A similar scenario was already presented in a model of systemic autoimmune disease and EAE (Lohr, Knoechel et al. 2006; Suryani and Sutton 2007). Specifically, it was shown that early during disease onset all three populations were present, with higher amounts of IFN-γ+/IL-17+ cells. Over time, the continuous decrease of IL-17 and double producing cells was compensated by the rise of IFN-γ+/IL-17- cells until disease stabilization. In this context, the authors suggested that the IFN-γ+/IL-17+ cells could be a pre-Th1 intermediate subset that further differentiates into an exclusively IFN-γ-producing population. Therefore, it could be argued that the variable IFN-γ/IL-17 cytokine proportions seen when analysing the skin of different psoriatic patients reflected the stage of disease progression at the point were the biopsy was taken. However, the double producing cells (IFN-γ+/IL-17+) could also represent already differentiated Th-17 cells, able to produce both cytokines due to a particular environment or situation, a scenario already seen for cells producing both IL-4 and IFN-γ (Acosta-Rodriguez, Rivino et al. 2007). Finally, consistent with published works (Langrish, Chen et al. 2005; Park, Li et al. 2005), IL-17 was mainly produced by memory T cells. In the blood IL-17 was secreted by both central and effector memory cells, while in the skin Th-17 cells were confined to the effector memory subset.

To explore if the increased IL-17 concentrations seen in the skin of psoriatic patients were directly responsible for disease pathogenesis, the effects of IL-17

blockade was investigated in the AGR psoriasis mouse model. Injection of IL-17 mAbs did not block T cell expansion and psoriasis development seen in the xenotransplantation mouse model. Instead, the use of anti-TNF-α, the "gold standard" in psoriasis treatment, specifically inhibited psoriasis development and T cell proliferation. These results confirmed previous work performed on a mouse model where epidermal hyperplasia was induced by injecting IL-23 in mice skin (Chan, Blumenschein et al. 2006). Also in this mouse model pre-treatment with anti-IL17 did not ameliorate epidermal hyperplasia. Additionally, it was shown that the direct injection of IL-17 into murine skin had minimal effects on epidermal hyperplasia. These results demonstrate that even though Th-17 cells may play an important role in psoriasis, IL-17 on its own is not a "master cytokine" in psoriasis pathology and that other effector mechanisms may synergize with IL-17 for disease outcome. A similar scenario has also been suggested in a model of T-cell-mediated colitis (Hue, Ahern et al. 2006; Kullberg, Jankovic et al. 2006). Here, it was shown that intestinal inflammation was associated with increased development of pathogenic Th-17 cells, however the neutralization of IL-17 alone was not enough to attenuate the disease. Interestingly, a significant amelioration of colitis was observed with anti-IL-6 and anti-IL-17 combination therapy, suggesting that both cytokines participate to the IL-23-driven intestinal inflammation. As discussed above for psoriasis, also during intestinal inflammation concomitant with the high levels of IL-17 there was a strict increase of IFN-γ, indicating that Th-17 and Th1 responses may synergize to elicit maximal pathologic effects. Based on these data it can be argued that psoriasis and intestinal inflammation present a similar pathological scenario that differs from that seen in the EAE and CIA model. In these latter animal models it has been proposed that the major pathogenic cell population might be the IL-17-secreting T cells alone, whereas Th1 cells, by secreting IFN-γ might rather inhibit the Th-17 subset (Harrington, Hatton et al. 2005; Park, Li et al. 2005; Mangan, Harrington et al. 2006). Based on these results it can be concluded that IL-17 is a critical cytokine in many autoimmune disease, but that its contribution during disease development varies for each disorder. In some diseases, like EAE, IL-17 may account for most of the pathological effects downstream of IL-23, but in other disorders like psoriasis and intestinal inflammation the blockade of IL-17 alone is not sufficient to inhibit disease onset and additional factors may also participate to the inflammatory reaction. In this

context, IL-22 has recently been proposed to be a major player in the pathogenesis of psoriasis. This cytokine is secreted by Th-17 cells and is involved in the proliferation and differentiation of keratinocytes (Wolk, Witte et al. 2006). High levels of IL-22 were found in the blood and in the diseased skin of patients with psoriasis. Interestingly, when comparing the cytokine amounts of psoriatic patients with healthy donors the differences in IL-22 levels were greater then the ones seen for other cytokines such as IFN-γ, IL-1 or IL-23 (Wolk, Kunz et al. 2004). Recently, it has also been shown that IL-22 is responsible of the acanthosis and dermal inflammation observed in the mouse skin after intradermal injection of IL-23 (Zheng, Danilenko et al. 2006). Therefore, theses results suggest that the increased number of Th-17 cells observed in psoriasis may exert their pathogenic effect by the release of both IL-17 and IL-22 and that these two cytokines may then synergize to regulate local tissue inflammation. A possible scenario could be that the release of IL-17 by Th-17 cells would induce the production of proinflammatory cytokines and chemokines by different cellular targets, including epithelial cells, endothelial cells and macrophages and thereby recruiting inflammatory cells to the damaged tissue (Acosta-Rodriguez, Rivino et al. 2007; Weaver, Hatton et al. 2007). IL-22, instead, would induce epidermal acanthosis through STAT-3 activation in keratinocytes, a pathway already shown to be critical for psoriasis development (Sano, Chan et al. 2005).

In conclusion this study suggests that the development of a psoriatic lesion depends on the presence of both IFN-γ and IL-17 cells and potentially other pro-inflammatory cytokines that act in concert in order to produce the typical tissue inflammatory reaction seen in psoriasis. The exact contribution of each T helper cell populations to this skin pathology remains to be determined. Thus, further work is required to delineate the effects of the IL-12/IFN-γ versus IL-23/IL-17 axis in the pathogenesis of psoriasis.

Chapter 4

Functional importance of integrin expression during the development of psoriasis

4. Functional importance of integrin expression during the development of psoriasis

4.1. Introduction

During an immune and inflammatory responses, the interaction of immune cells with other cells and with the extracellular matrix (ECM) is mediated by adhesion molecules. These molecules are mainly transmembrane proteins, which provide a link between cells and their environment and are of fundamental importance to many cellular functions such as regulation of cell motility, gene expression and differentiation (Parks 2007). The loss of adhesive interactions as well as an aberrant expression of adhesion molecules may result in disease states (e.g. pemphigous vulgaris, leukocyte adhesion deficiency-1, arteriosclerosis, diabetic vasculopathy) (Petruzzelli, Takami et al. 1999). Cell adhesion molecules can be classified in 4 major groups which include integrins, cadherins, selectins and members of the immunoglobulin superfamily (Petruzzelli, Takami et al. 1999). Among these four major groups the one with highest affinity to ECM components is the integrin group. Integrins are a large family of transmembrane heterodimeric glycoproteins containing two non-covalently associated α and β subunits, which combine to form more then 20 different receptors (Pozzi, Wary et al. 1998). A single β chain can interact with different α chains, forming integrins that bind different ligands. Integrins relay information via out-side-in signalling and regulate a vast array of cellular functions including proliferation, migration, gene expression and survival (Watt 2002). Therefore, they are involved in tissue homeostasis and play a critical role during injury, infection and inflammation (Parks 2007). The importance of these proteins is further stressed by the dramatic consequences of genetics defects in the integrins genes. For example, diminished or absent expression of the β_2 integrin chain lead to a disease called leukocyte adhesion deficiency-1 (LAD-1). Patients bearing this mutation suffer from impaired wound healing with high risk of bacterial infections. Similarly, mutations in the α or in the β subunit of the gpIIbβ_3 integrin increase the susceptibility to bleeding, as seen in patients with Glanzmann thrombasthenia (Petruzzelli, Takami et al. 1999). An aberrant expression of integrins can, on the

other hand, induce pathological conditions. During the formation of an atherosclerotic plaque one pathological event is the dramatic increase in platelet adhesiveness. Antibodies directed against platelets integrins have been shown to ameliorate atherosclerotic vascular changes (Petruzzelli, Takami et al. 1999).

In skin, the most prevalent integrins belong to the β_1 subfamily. This group includes adhesion molecules with high affinity for many ECM proteins such as fibronectin, collagens and laminins. In healthy skin, β_1 integrin expression is confined to the basal layer and has a fundamental role in anchoring KCs to the basement membrane. The loss of integrin β_1 expression at this level leads to blistering and failure in basement membrane organisation (Raghavan, Bauer et al. 2000). A suprabasal integrin expression is a feature of hyperproliferative epidermis and is seen during wound healing or psoriasis (Watt 2002).

Collagens are the most abundant proteins in the ECM and they account for 75% of skin's dry weight (de Fougerolles, Sprague et al. 2000). The major cell surface receptors for collagen are the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. $\alpha_1\beta_1$ preferentially binds collagen IV, the major component of the basement membrane and is mainly expressed on mesenchymal cells, fibroblasts, hepatocytes and endothelial cells. $\alpha_2\beta_1$ shows a preference for binding to collagen I and is preferentially expressed in the epithelial compartment (de Fougerolles, Sprague et al. 2000). Both integrins show very low expression on peripheral blood lymphocytes. However long term activation of T cells induces expression of both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, consistent with the initial description of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ as very late antigens (VLA). Finally, both integrins were shown to play a role in cell migration (Dustin and de Fougerolles 2001), and in reorganization and contraction of collagen fibres during wound healing (Schiro, Chan et al. 1991).

Psoriasis is a skin disorder characterized by epidermal hyperplasia, inflammation and neoangiogenesis. Two of the most prominent phenotypical changes during the formation of a psoriatic lesion are the increased turnover of KCs and the intense infiltration of activated T cells in the epidermal compartment. The fact that suprabasal integrin expression is a characteristic of hyperproliferative epidermis and that infiltrating T cells in chronically inflammatory diseases express $\alpha_1\beta_1$, suggested that integrins may be of importance in psoriasis. Based on these two observations a

closer analysis of integrin expression, in particular $\alpha_1\beta_1$ and $\alpha_2\beta_1$, during the development of psoriasis was performed. The first part of this chapter will be dedicated to the relationship of $\alpha_1\beta_1$ expression on effector T cells and the development of psoriasis. The second part will refer to the functional role of $\alpha_2\beta_1$ expression in the epidermal compartment of psoriatic lesions.

4.2. A critical role for $\alpha_1\beta_1$ (VLA-1) in accumulation of epidermal T cells and the development of psoriasis

The aetiology of autoimmune inflammatory disorders such as rheumatoid arthritis, Crohn's disease and psoriasis is complex and many aspects are still unclear. A common characteristic of these diseases is a permanent inflammatory process within lymphoid organs and involved tissue. In the last years, increasing number of studies suggested a strong correlation between the expression of $\alpha_1\beta_1$ on tissue infiltrating T cells and the development of chronic inflammatory disorders (de Fougerolles, Sprague et al. 2000). In particular, the importance of $\alpha_1\beta_1$ integrin was demonstrated in animal models of hypersensitivity, arthritis and experimental colitis.

$\alpha_1\beta_1$ is a major cell surface receptor for collagen with a preference for collagen IV. $\alpha_1\beta_1$ is expressed on mesenchymal cells, including smooth muscle cells, fibroblasts and microvascular endothelium (Krieglstein, Cerwinka et al. 2002). Naïve T cells express only low levels of $\alpha_1\beta_1$, whereas long term activation (4-6 weeks) significantly increases the levels of this integrin, hence the alternative name VLA-1 (very late antigen- 1). Finally, $\alpha_1\beta_1$ positive T cells belong to a restricted subset of lymphocytes, the memory T cells, and are all IFN-γ producing T cells (Goldstein, Ben-Horin et al. 2003). Given the functional importance of the ECM environment during the migration of T cells in a psoriatic lesion and the fact that chronic activated T cells express the VLA-1 receptor, the role of $\alpha_1\beta_1$ function during the onset of psoriasis was investigated. In the first part (results 1) of this chapter, a summary of the most relevant results, performed by other member of our group, will be presented. The main focus of this part are the relationship of epidermal accumulation of $\alpha_1\beta_1$ memory

Th1 cells and psoriasis onset, and the prevention of psoriasis development by blocking $\alpha_1\beta_1$ with a monoclonal antibody in a clinically relevant psoriasis mouse model. In the second part (results 2), a more detailed description of the experiments performed during my PhD thesis that also complement the previous part will be shown.

4.2.1. Results 1

4.2.1.1. Exclusive expression of $\alpha_1\beta_1$ integrin on epidermal but not dermal T cells

Based on the fact that in inflammatory skin disorders infiltrating T cells express $\alpha_1\beta_1$ integrins (de Fougerolles, Sprague et al. 2000), the first step was to investigate the expression of this receptor on psoriatic T cells. Staining of psoriatic skin sections with an anti-α_1 mAb revealed the presence of $\alpha_1\beta_1$ on leukocytes in the epidermis but not dermis of lesional psoriatic skin (Figure 4.1A). In contrast, mononuclear cells positive for $\alpha_1\beta_1$ could not be detected either in uninvolved skin from psoriatic patients or in the skin of healthy donors (data not shown). Dual colour immunofluoresence was then performed to better characterise the VLA-1+ cell population (Figure 4.1B and C). 20-50% of epidermal infiltrating CD3+ cells (*red*) co-expressed $\alpha_1\beta_1$ integrin *(green*), while no α_1-integrin could be detected on dermal T cells. $\alpha_1\beta_1$ positive cells observed in the dermis represent mesenchymal cells such as smooth muscles cells and vascular endothelium. A closer analysis of $\alpha_1\beta_1$ epidermal T lymphocytes demonstrated that these cells were predominantly effector memory T cells with a type 1 cytokine profile. In fact, when cells were freshly isolated from lesional psoriatic skin biopsies most of the $\alpha_1\beta_1$/CD3+ T cells were CCR7-/CD45- (Figure 4.1E), a phenotype compatible with effector memory cell differentiation (Sallusto, Geginat et al. 2004). Moreover, intracellular cytokine staining showed that the majority of epidermal CD3+ cells secreted IFN-γ but not IL-4, and that nearly all IFN-γ+ cells expressed $\alpha_1\beta_1$ integrin receptors (Figure 4.1F-H).

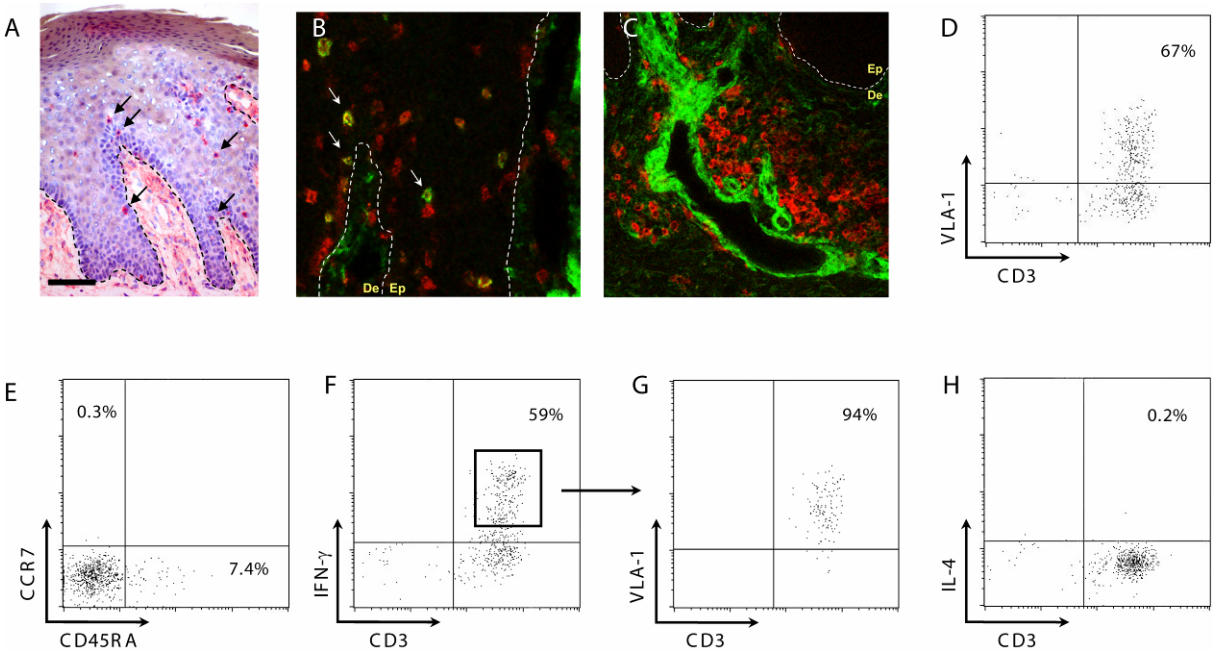

Figure 4.1 Expression of $\alpha_1\beta_1$ on human epidermal but not dermal T cells in psoriasis. (A) Cryosesctions of lesional psoriatic skin were stained for α_1 integrin and revealed the presence of $\alpha_1\beta_1$ positive cells only in the epidermal compartment (black arrows). (B and C) Two-colour immunofluoresence staining of psoriatic lesions with α_1-integrin (green) and CD3 (red). (B) Several epidermal T cells appeared yellow as a consequence of co-localization of CD3 and $\alpha_1\beta_1$ (white arrow). (C) Green dermal staining reflects the expression of $\alpha_1\beta_1$ on mesenchymal cells. (D-H) Flow cytometry analysis of epidermal lymphocytes freshly isolated from the epidermis of lesional psoriatic skin. (D) On average 63% +/- 6.2% of CD3+ cells co-expressed the $\alpha_1\beta_1$ integrin receptor (n=5). (E) The majority of epidermal CD3+/$\alpha_1\beta_1$ cells were CCR7-/CD45RA-, indicating an effector memory phenotype.(F-G) Flow cytometry analysis (n=3) demonstrated that almost 60% of epidermal T cells secreted IFN-γ and that nearly all of these cells were $\alpha_1\beta_1$+ . Not any CD3+ cell produced IL-4 (H). Scale bar in A represent 50μm. Dashed line indicate border between epidermis above and dermis below. Ep, epidermis; De, dermis. Dot plots shown are gated on lymphocytes.

4.2.1.2. Migration of T cells into epidermis parallels psoriasis onset

In order to study the expansion of T cells during the development of a psoriatic lesion, a time course experiment was performed. Uninvolved human psoriatic skin was transplanted on the AGR xenotransplantation mouse model and skin sections were obtained after 0, 7, 21 and 35 days. Sections were stained for CD3 and T cell expansion was analysed. At day 0 no T cells were present in the epidermis and only few T cells could be detected in the dermis. 7 days later an accumulation of dermal CD3+ T cells took place, while the epidermis remained unaltered. At day 21 the entrance of dermal T cells into the epidermis correlated with a thickening of the epidermis (acanthosis) and some papillomatosis. At day 35, beside numerous epidermal T cells, the typical histological signs of psoriasis, acanthosis and papillomatosis, were evident. Taken together this experiment revealed an early

proliferation of T cells in the dermis followed by a considerable migration into the epidermis (Figure 4.2A). Interestingly, dermal T cells (*dashed line*) showed marked proliferation until day 14 and thereafter a progressive reduction, while in the epidermal compartment only around day 21 the first T cells could be detected (*solid black line*). From these results it could be concluded that in developing psoriatic lesions expansion of dermal T cells precedes typical psoriasisform changes and that accumulation of epidermal T cell correlates with the development of psoriatic epidermal hyperplasia (Figure 4.2B).

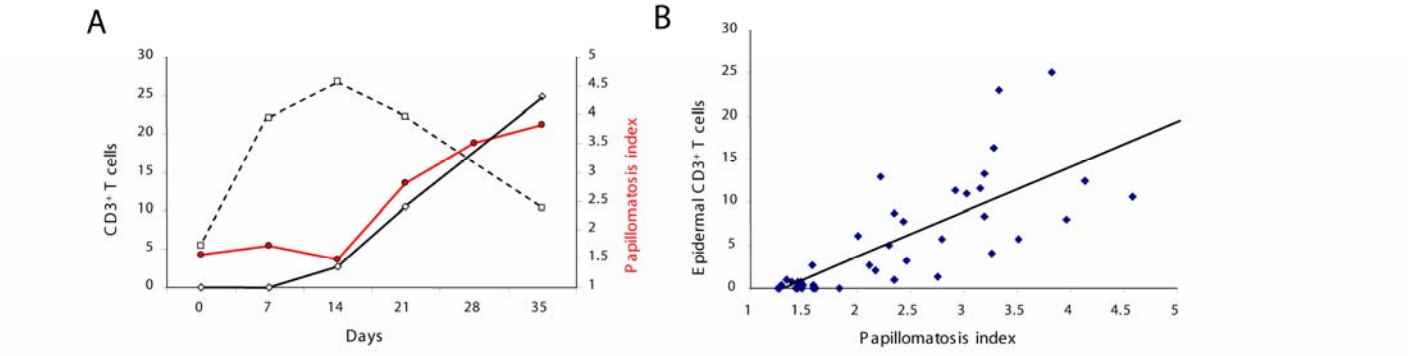

Figure 4.2 Expansion of epidermal but not dermal T cells correlates with epidermal psoriasisform changes. (A) Quantification of T cell expansion on grafts transplanted on the AGR mouse model (d0, d7, d14, d21 n=2, d35 n=1). In contrast to the number of dermal T cells (dashed line) the expansion of epidermal CD3+ T cells (solid black line) parallels the onset of histological psoriatic features represented by the papillomatosis index (red line). (B) Significant correlation of epidermal CD3+ cells and papillomatosis index. Data points shown (n=41) represent grafts of different PN skin, PN skin with active immune compartments, and psoriasis developing PN skin treated or untreated taken at different time points including d0, d7, d14, d21, d28 and d35. Data shown in A are representative of two independent experiments with PN skin from two different psoriatic patients.

4.2.1.3. Effects of $\alpha_1\beta_1$ blockade in vitro and in vivo

The basement membrane, which separates the dermis from the epidermis, is rich in collagen IV, the main ligand for $\alpha_1\beta_1$. Therefore, when T cells migrate into the epidermal compartment they come in contact with these collagen fibers. To test if T cells need $\alpha_1\beta_1$ integrin to attach and migrate through this structure an *in vitro* transmigration assay was performed. Peripheral T cells were cultured with phytohemagglutinin (PHA) plus IL-2 and as soon as $\alpha_1\beta_1$ expression exceeded 60% (after approx. 4-6 weeks), T cells were isolated by depletion of non-T cells. Migration of $\alpha_1\beta_1$ T cells trough collagen IV-coated transwells, using SDF-1α (stromal cell-derived factor 1 or CXCL12) as chemoattractant was significantly blocked by adding anti-α_1 mAbs compared to isotype control mAb (Figure 4.3A).

To determine the consequences on T cell migration after blocking $\alpha_1\beta_1$ in vivo, uninvolved psoriatic skin was transplanted on AGR mice and treated with either anti-human anti-α_1 (n=7) or isotype control mAbs (n=5). CD3+ T cells were quantified in skin grafts excised 35 days after transplantation and stained with anti-CD3 mAb. Treatment with anti-α_1 mAb almost completely inhibited expansion of epidermal T cells compared to skin grafts treated with isotype control mAb. The number of epidermal T cells found in the transplants of anti-α_1 treated mice were comparable to that counted in non-lesional pre-psoriatic skin (PN) from the same psoriatic patient (Figure 4.3B).

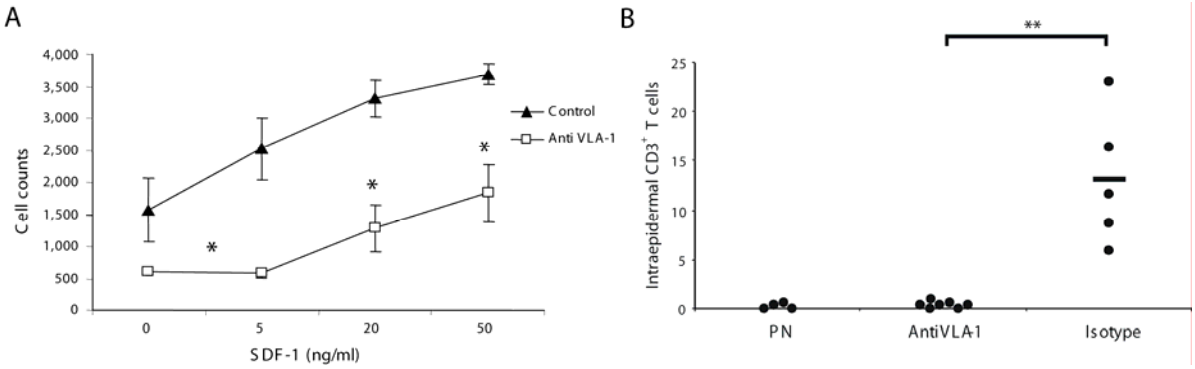

Figure 4.3 $\alpha_1\beta_1$ blockade inhibits T cell migration through collagen IV matrices *in vitro* and accumulation of epidermal T cell *in vivo*. (A) Migration of cultured T cells trough collagen IV-coated transwells using SDF-α as a chemoattractant. Numbers of T cells were measured by flow cytometry analysis of each sample counted for 3 minutes. Data graphed represent mean values +/- standard error of the mean (SEM) deviation for SDF-1α at 0, 5, 20 and 50 ng/ml. Depicted migration assay was performed in triplicates and is representative for data obtained from three independent experiments. (B) Counts of epidermal T cells in human skin sections taken 35 days after transplantation on AGR mice. Injection of anti-α_1 mAb significantly reduced the number of epidermal T cells compared to isotype control treatment. *, $p<0.05$; **, $p=0.0005$

Anti-α_1 treatment was also efficacious in inhibiting the development of psoriatic lesion, in 7 out of 7 mice grafted with uninvolved skin from three different psoriatic patients. Histological evaluation of grafts injected with anti-α_1 mAb revealed significant reduction of both acanthosis and papillomatosis index compared to isotype control mAb (Figure 4.4A). Moreover, the treatment with anti-α_1 mAb was as effective as the blockade with anti-TNF-α mAb, the current "gold standard" of anti-psoriatic therapy. Taken together, these data demonstrate that blocking the interference of $\alpha_1\beta_1$ integrin with collagen IV inhibits the migration of T cells into the epidermis and thereby prevents the development of psoriatic lesions (Conrad, Boyman et al. 2007).

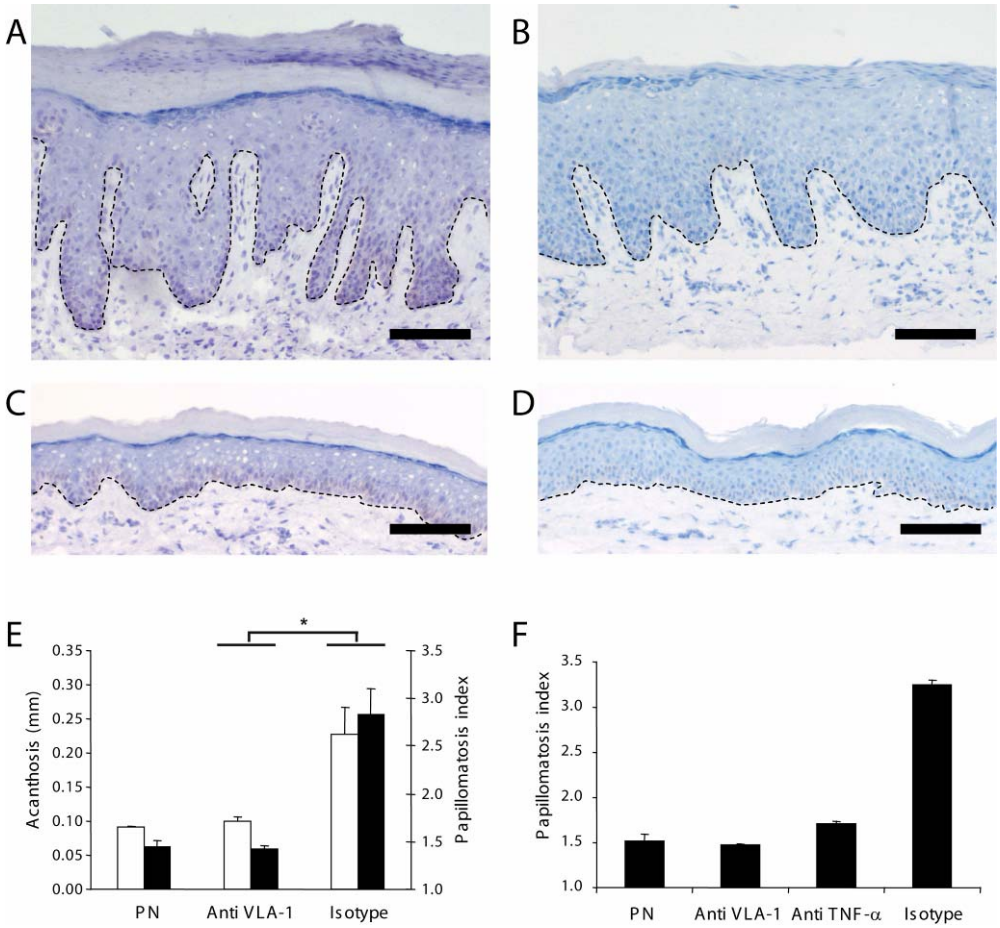

Figure 4.4 Blocking α_1-integrin inhibits psoriasis development. (A) Uninvolved psoriatic skin transplanted on AGR mice developed fully-fledged psoriasis 35 days after engraftment and treatment with isotype control mAb (representative of n=5) and presented same histological features as lesional psoriatic skin of the same patient (B). (C) Graft treated with anti α_1-integrin mAb (representative of n=7) presented a histological picture indistinguishable from uninvolved psoriatic skin on day 0 of the same patient (D). (E) Acanthosis (open bars) and papillomatosis (solid bars) indices on day 0 and 5-6 weeks after transplantation on AGR mice treated either with anti-α_1 (n=7) or isotype control mAb (n=5). (F) Treatment with anti-α_1 mAb induced a reduction of the papillomatosis index comparable to the treatment with anti-TNF-α (n=2). Scale bares (A-D) represent 100μm. Dashed line (A-D) indicates border between epidermis above and dermis below. Data depicted represent mean values plus standard error of the mean (SEM) (E), and mean values of triplicates (F). *, $p \leq 0.003$

4.2.2. Results 2

4.2.2.1. Characterisation of intraepidermal $\alpha_1\beta_1$ T cells

In the previous section it has been shown that $\alpha_1\beta_1$ was exclusively expressed on IFN-γ T cells in the epidermis and that these cells were effector memory T lymphocytes (CCR7-/CD45RA-). Subsequent immunofluoresence staining performed on lesional psoriatic skin sections, revealed that the majority of $\alpha_1\beta_1$+/CD3+

epidermal T cells belong to the CD8+ subset, and only few of them were CD4+ (Figure 4.5A-D). Identical results were seen when immunofluorescence staining was performed on grafts excised after development of the psoriasiform lesion (d35). Again CD3+ T cells were expressing $\alpha_1\beta_1$ integrin and were mainly of the CD8+ phenotype (Figure 4.5E and F).

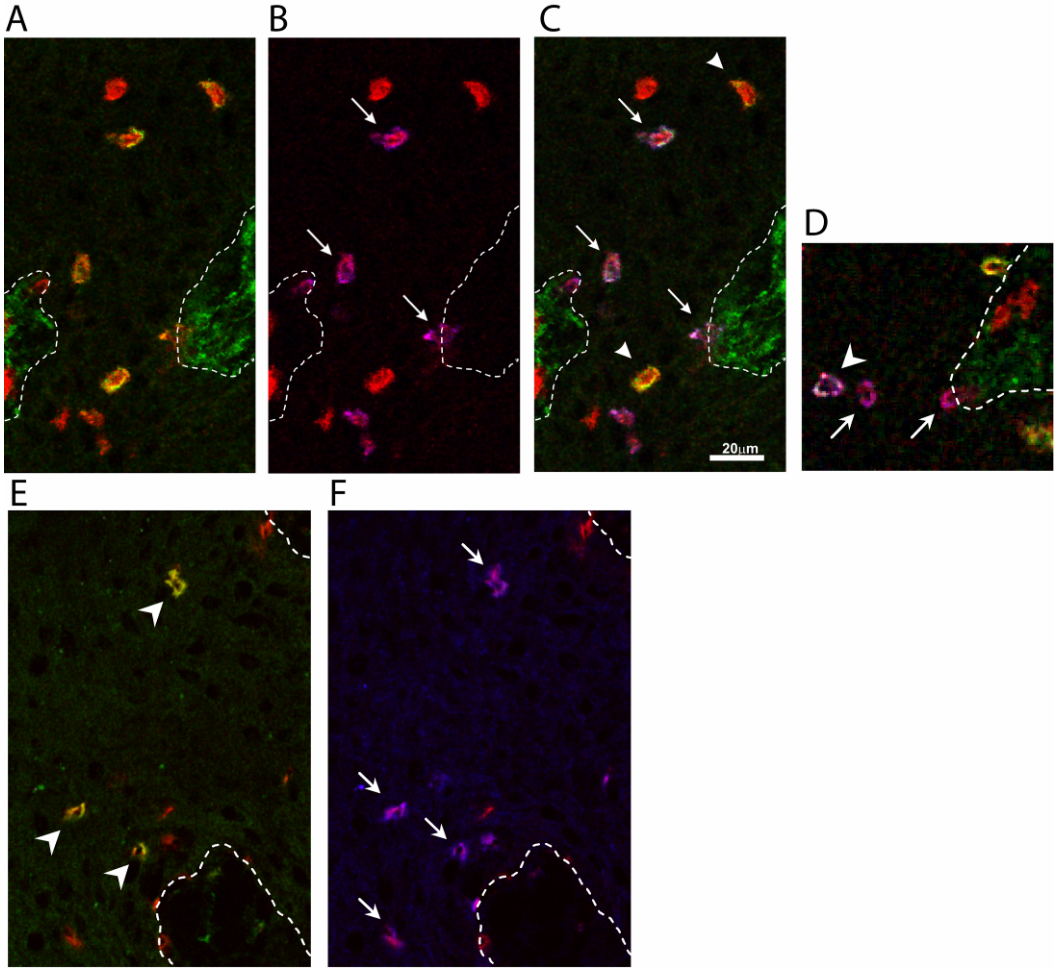

Figure 4.5 CD3+/CD8+ and CD3+/CD4+ epidermal T cells express α_1 integrin. Three-color immunofluorescence staining using FITC-conjugated mAb to CD49a (α_1) (green), PE-conjugated mAb to CD3 (red), and APC-conjugated mAb to CD8 (A-C) or CD4 (D) (blue) was performed on lesional psoriatic skin. (A) Several epidermal T cells appeared in yellow as a consequence of co-localization of CD3 and $\alpha_1\beta_1$. (B) The majority of epidermal T cells showed co-localization of CD3 and CD8 and therefore, appeared in magenta (white arrows). (C) A subpopulation of both epidermal CD8+ (white arrows) and CD8- (yellow, arrow heads) CD3+ T cells expressed $\alpha_1\beta_1$. (D) A subpopulation of CD4+/CD3+ T cells (magenta, white arrows) appeared in white (arrow heads) as a consequence of co-localization of CD3, CD4, and $\alpha_1\beta_1$. CD4-/CD3+ epidermal T cells representing CD8+ T cells appeared in yellow showing expression of α_1 integrin. (E and F) Grafts excided (d35) after development of a psoriasiform phenotype in the AGR mouse model. (E) Epidermal T cells appeared in yellow as a consequence of co-localization of CD3 and $\alpha_1\beta_1$ (yellow, arrow heads). (F) The majority of epidermal T cells in xenografts showed co-localization of CD3 and CD8 and therefore appeared in magenta (white arrows). Location of several $\alpha_1\beta_1$ expressing epidermal T cells (e, arrow heads) corresponded to CD8+/CD3+ T cells (f, white arrows) indicating co-expression of $\alpha_1\beta_1$/CD8/CD3. Dashed line (a-f) indicates border between epidermis and dermis.

In order to even better characterise this intraepidermal subpopulation of T cells, flow cytometry analysis was performed on cells freshly isolated from lesional psoriatic skin of psoriasis patients. Epidermal T cells were stained with mAb to CD3 and to $\alpha_1\beta_1$ in conjunction with other antibodies against different surface markers. These analyses showed that the majority of $\alpha_1\beta_1$+/CD3+ T cells were CD8 positive (Figure 4.6B), thereby confirming the immunofluorescence results, and that almost no cells were positive for Vγ9 and Vδ1, two markers present on unconventional $\gamma\delta$ T cells (Figure 4.6C and D). Further staining demonstrated that a significant number of intraepidermal $\alpha_1\beta_1$+ T lymphocytes also expressed the $\alpha_E\beta_7$ integrin (CD103), which mediates the adhesion to E-Cadherin on keratinocytes and favours the retention of T cells in the epidermis (Figure 4.6E). Another receptor highly expressed on intraepidermal α_1+ T cells was NKG2D, a C-type lectin like protein normally expressed on NK cells, CD8+ T cells and $\gamma\delta$ T cells (Saez-Borderias, Guma et al. 2006); whereas no expression of the skin-homing-associated receptor CCR4 could be detected (Figure 4.6F and G). The analysis of the total epidermal cell suspension revealed that no $\alpha_1\beta_1$+/CD56+ NK cells were present in the epidermis of psoriatic patients (Figure 4.6H).

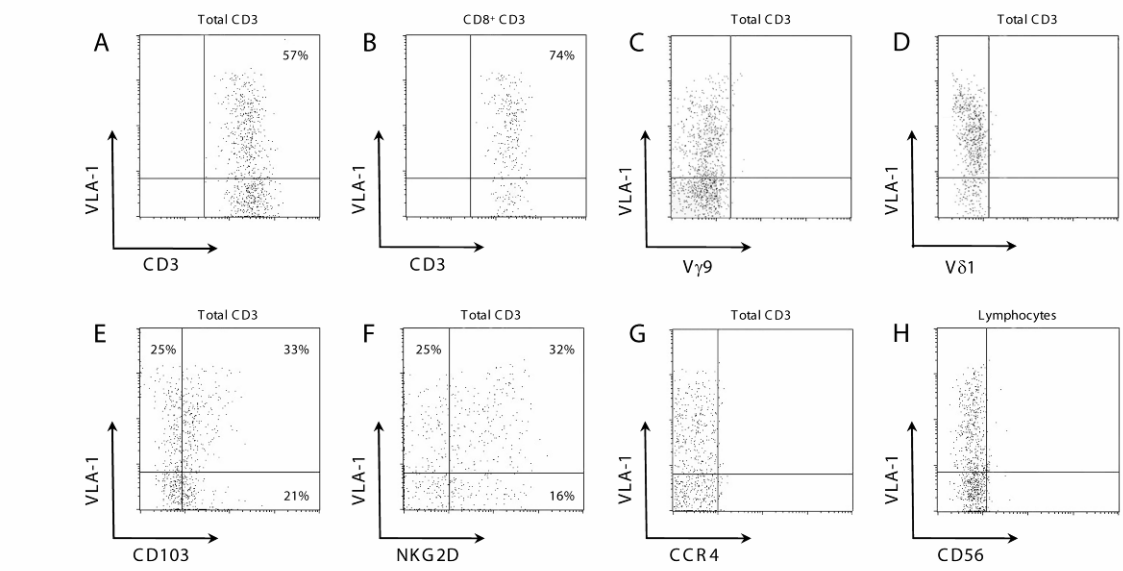

Figure 4.6 Extended Phenotype of freshly isolated $\alpha_1\beta_1$+ psoriatic T cells. Flow cytometry analyses of epidermal T cells freshly isolated from lesional psoriatic skin. (A and B) Approximately 60% of epidermal CD3+ cells and even 75% of the CD8+ subpopulation were also positive for $\alpha_1\beta_1$ integrin. The expression of Vγ9 (C) and Vδ1 (D), was barely or not detectable on CD3+/$\alpha_1\beta_1$+ epidermal T cells. Further analysis of the $\alpha_1\beta_1$ population revealed that the majority of intraepidermal CD3+ cells were expressing CD103 (E) and NKG2D (F), whereas no expression of the skin-homing marker CCR4 (G) could be detected. Analysis of the total epidermal lymphocyte population demonstrated that almost no $\alpha_1\beta_1$+/CD56+ cells were present in the epidermis of lesional psoriatic skin (H). Data depicted are representative of 3 independent experiments.

4.2.2.2. Contact with collagen IV induces expression of $\alpha_1\beta_1$ integrin on T cells

Epidermal T cells but not dermal T lymphocytes seem to express the $\alpha_1\beta_1$ receptor. This implies that collagen IV present in the basement membrane of the skin might induce the up-regulation of this integrin, when T cells emigrate from the dermis to the epidermis. This hypothesis was confirmed by an *in vitro* transmigration assay using freshly isolated human T cells. $\alpha_1\beta_1$ expression on CD3+ cells was measured before and after 4 hours migration through collagen IV-coated transwells using SDF-1α (CXCL12) as a chemoattractant (Figure 4.7). Interestingly, percentage of $\alpha_1\beta_1$ positive T cells increased significantly after migrating through collagen IV as compared to the expression at the beginning of the experiment. These data demonstrate that the interaction of T cells with collagen IV induces the expression of $\alpha_1\beta_1$ integrin on their surface.

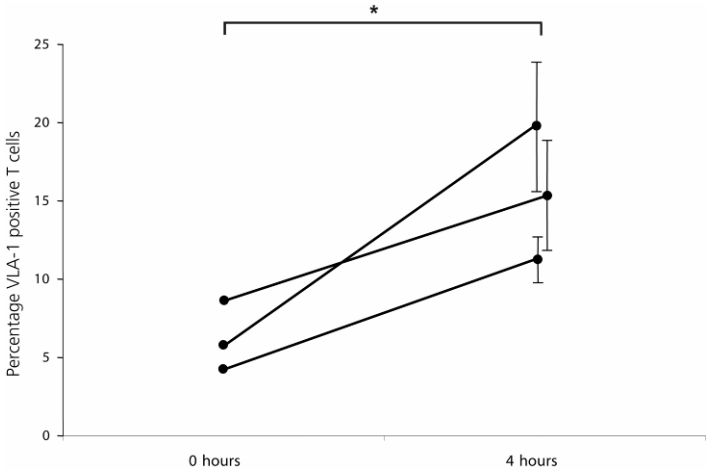

Figure 4.7 Collagen type IV increases $\alpha_1\beta_1$-expression on T cells in vitro. Percentage of $\alpha_1\beta_1$ expression of freshly isolated T cells before and after migration through collagen IV-coated transwells using SDF-1α (CXCL12) as a chemoattractant. Graph displays three independent experiments with T cells from three different healthy donors. Migration assays were performed in triplicates. Data points t=4h represent mean values of triplicates +/- standard deviation (SD) for SDF-1α at 50ng/ml after 4 hours. *, $p<0.01$

4.2.2.3. Anti-α_1 mAb treatment does not induce T cell apoptosis

In paragraph 4.2.1.2 it was shown that treatment with anti-α_1 mAb significantly reduces the number of CD3+ T cells in the AGR xenograft mouse model. This epidermal T cell reduction could also be attributed to non-specific cytotoxic effects of the antibody. In order to address this hypothesis an in vitro test evaluating the direct

effect of anti-α_1 mAb on T cells was performed. $\alpha_1\beta_1$+ T cells, obtained as already described in paragraph 4.2.1.3, were incubated at various concentrations of anti-α_1 mAb and different time-points. Percentage of cells undergoing apoptosis was measured by flow cytometry after staining them with annexin V and propidium iodide (PI) (Figure 4.8C-E). While irradiation with single dose of 4200 rad readily induced T cell apoptosis, none of the indicated concentration of monoclonal antibody to $\alpha_1\beta_1$ led to an increase of apoptosis as compared to isotype control antibody or medium alone (Figure 4.8A and B). Moreover cell apoptotic rate did not increase with longer incubation times. Based on these results it could be concluded that anti-α_1 mAb blocks the emigration of dermal T cells into the epidermis, rather than inducing a cytotoxic effect on $\alpha_1\beta_1$+/CD3+ T cells.

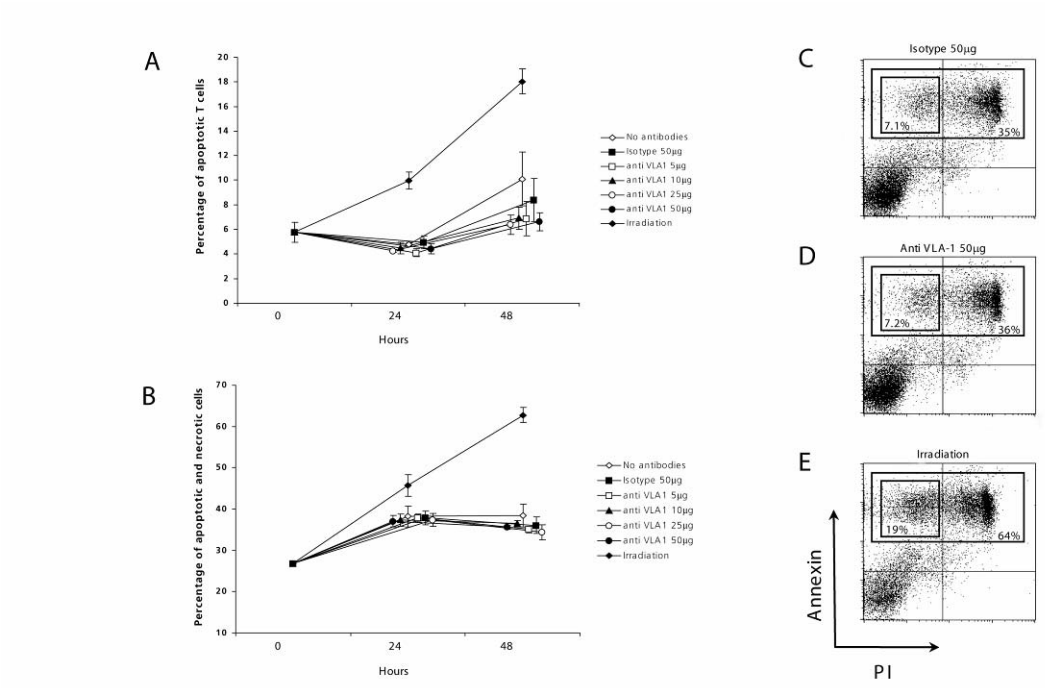

Figure 4.8 No induction of apoptosis via monoclonal antibody to α_1. Graphs A and B show percentage of T cells undergoing early apoptosis (cells single positive for annexin V) and percentage of total early and late apoptotic cells and necrotic cells (cells double positive for PI and annexin V) respectively. Figure C, D and E are representative for data obtained from 2 independent experiments. Early apoptotic cells staining positive for annexin, but not PI, appear in the upper left quadrant of dot plots. Late apoptotic/necrotic cells appear in the upper right quadrant, staining positive for both PI and annexin V. Double negative staining represents live cells. Small boxes (C-E) represent percentage of early apoptotic T cells, large boxes (C-E) represent percentage of total apoptotic and necrotic T cells.

4.2.2.4. Effect of anti-$\alpha_1\beta_1$ treatment on lesional psoriatic skin.

The final step of this study was to investigate the efficacy of $\alpha_1\beta_1$ treatment on fully developed psoriatic skin transplanted on AGR mice. Injection of anti-α_1 mAb

(n=4) only slightly reduced the acanthosis (data not shown) and papillomatosis index as compared to isotype control (n=2) or skin prior to transplantation (n=3) (Figure 4.9). In contrast, anti TNF-α therapy was still able to significantly reduce epidermal hyperplasia. An explanation for these results may be in the fact that anti-α_1 mAb prevents the development of a psoriatic lesion by inhibiting the migration of dermal T cells into the epidermis. Therefore, in an established psoriatic lesion, where a significant number of T cells are already present in the epidermis, $\alpha_1\beta_1$ blockade is not able to ameliorate the disease. However, in our previous study (Conrad, Boyman et al. 2007), when psoriatic skin harbouring an active immune compartment (defined by increased numbers of dermal T cells with few T cells already present in the epidermis) was transplanted on AGR mice, anti-α_1 treatment could still show an inhibitory effect on the development of psoriasis. In fact, $\alpha_1\beta_1$ blockade inhibited the emigration of new T cells into the epidermis and few T cells already present in the epidermal compartment were not sufficient to induce psoriasisform changes. Subsequent staining with anti-CD3 mAb clearly showed the effect of the anti-α_1 treatment. Although total T cell numbers remained unchanged in all grafts and throughout all the experiment, a difference in the distribution of these cells was observed. Anti-α_1 treated grafts presented an accumulation of T cells beneath the basal membrane with almost no cells in the epidermis. In contrast, isotype control treated grafts showed a significant reduction of dermal T cells and instead an increase of epidermal T lymphocytes. Thus, this experiment confirmed the effect of anti-α_1 mAb in inhibiting the movement of resident T cell in the epidermis and excluded its putative cytotoxic effect.

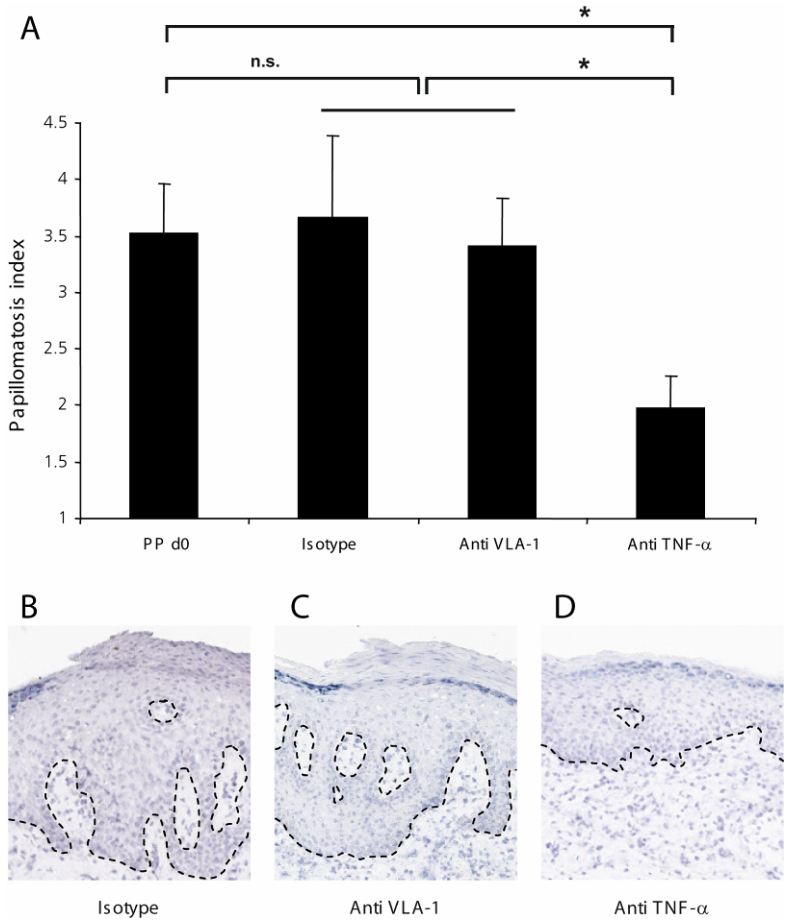

Figure 4.9 $\alpha_1\beta_1$ blockade in established psoriatic skin. (A) Papillomatosis index of lesional psoriatic skin 3 weeks after transplantation onto AGR mice and injected with either isotype control mAb (n=2), mAb to α_1 (n=4) (anti VLA-1) or mAb to TNF-α (n=3). (B-D) Microscopic view of PP skin 3 weeks after transplantation onto AGR mice treated with isotype control (B), monoclonal antibody to α_1 (C), and monoclonal antibody to TNF-α, (infliximab) (D). Data depicted represent mean values plus standard deviation (SD) (a). Dashed line (B-D) indicates border between epidermis above and dermis below. *,$p<0.05$; n.s. not significant

4.2.3. Discussion

Experiments performed in section “Results 2” confirmed and extended the initial study regarding the critical role of $\alpha_1\beta_1$ integrin in the development of psoriasis. A more detailed characterisation of the $\alpha_1\beta_1$ epidermal T cell subpopulation could be given by analysing the expression of different surface markers. In addition of being effector memory Th1 cells, the majority of $\alpha_1\beta_1$+ T lymphocytes were shown to be CD8+, but not CD56, Vδ1 or Vγ9 positive. Moreover, a consistent number of $\alpha_1\beta_1$+ T cells was also expressing the integrin $\alpha_E\beta_7$, which is the ligand for KCs E-cadherin,

and therefore can possibly explain the retention of T cells in the epidermis. Based on these results a simplified two step scenario has been proposed (Conrad, Boyman et al. 2007). During their migration from the dermis to the epidermis, effector T cells would interact with collagen IV and therefore induce the expression of $\alpha_1\beta_1$ integrin. Once in the epidermis, T lymphocytes would up-regulate integrin $\alpha_E\beta_7$ and progressively downregulate $\alpha_1\beta_1$ expression. In fact, in the epidermis, in addition to $\alpha_1\beta_1$+/$\alpha_E\beta_7$+ T cells also $\alpha_1\beta_1$-/$\alpha_E\beta_7$+ cells have been observed. An additional experiment confirmed the critical role of collagen IV on $\alpha_1\beta_1$ T cell expression. In fact, when freshly isolated T cells migrated trough collagen IV coated transwells an increase in $\alpha_1\beta_1$ expression could be detected. Previous experiments performed on AGR mice showed that blockade of $\alpha_1\beta_1$ inhibited migration of T cells into the epidermis and prevented the development of a psoriatic lesion. The reduced number of T cells observed after treatment with anti-α_1 mAb was shown not to be the consequence of an unspecific cytotoxic effect of the Ab. In fact, incubation of $\alpha_1\beta_1$+ T cells with different concentration of anti-α_1 mAb did not induce any increase in the apoptosis rates if compared to isotype antibody or medium alone. While, T cells irradiated with a single dose of 4200rad, readily underwent apoptosis. A final experiment demonstrated that $\alpha_1\beta_1$ blockade was not able to to significantly ameliorate established psoriatic lesions in AGR mice. In fact, anti-α_1 mAb has been demonstrated to inhibit the development of psoriasiform lesions by blocking the migration of T cells in the epidermis. Therefore, in a situation where T cells are already present in the epidermis the mAb may not be effective anymore. Though, anti-α_1 mAbs, by significantly reducing the number of epidermal T cells, eliminate the supply of additional T cells needed for chronification of disease and thereby effectively improve psoriasis. In addition, a therapy with anti-α_1 mAb may be considered as a strategy in the long term to avoid disease relapses.

Taken together this work establishes $\alpha_1\beta_1$+ epidermal T cells as an important subset of effector memory lymphocytes in the development of psoriatic lesions. It also demonstrates that inhibition of α_1 integrin results the blockade of psoriasis development as a consequence of T cell migration blockade into the epidermis.

4.3. Blockade of $\alpha_2\beta_1$ integrin inhibits psoriasis in a T cell independent manner

Throughout this thesis psoriasis has been regarded as a T cell- mediated disease. T cells were shown to be crucial for the generation of psoriatic lesions and their migration into the epidermis was demonstrated to precede psoriasisform changes. However, it has to be kept in mind, that psoriasis is a skin disorder, with KCs hyperproliferation as one of the most evident phenotypical changes. In this context, several reports have already suggested KCs as potential initiator of inflammation, via production of cytokines, growth factors as well adhesion molecules important for the disease initiation. In 2005 Zenz et al. published an interesting study considering psoriasis as an epidermal disease. By knocking out in the epidermis two components of the AP-1 transcription factor (JunB and c-Jun) involved in cell proliferation and differentiation in the epidermis, they created a new animal model with psoriasis-like features (Zenz, Eferl et al. 2005). At the skin level these mice showed typical hallmarks of psoriasis such as hyperkeratosis, enlarged blood vessels, lymphocytic infiltration and up-regulation of several cytokines known to be increased in psoriatic lesions. By analysing the two JunB and c-jun components in humans, the authors observed that JunB was down-regulated and c-jun slightly up-regulated in the skin of psoriatic patients. Although many questions arose whether this mouse model truly resembles psoriasis, this work shifted again the interest towards KCs as initiator of psoriasis. However, it is still a controversial whether psoriasis arises from a primary alteration in epidermal KCs or in immunocytes. A reasonable compromise would be that the perpetuation of the inflammatory processes in psoriasis originates from the mutual interaction and activation between KCs and T cells. This aspect of psoriasis biology will be investigated in the last part of this thesis.

Attachment of KCs to the basement membrane is mediated by integrin receptors, including $\alpha_6\beta_4$ integrin, which is a receptor for laminin, and by several β_1 integrins, the most abundant of which are $\alpha_2\beta_1$ (collagen receptor) and $\alpha_3\beta_1$ (laminin receptor). In situations in which the epidermis is hyperproliferative, such as during wound healing and psoriasis, all three integrins have been shown to be expressed suprabasally.

Carroll et al generated transgenic mice in which human integrins β_1, α_2 and α_5 were expressed under the control of the involucrin promoter, thereby limiting their expression into skin (Carroll, Romero et al. 1995). Transgenic mice expressing the β_1 subunit alone or in combination with α_2 or α_5, presented typical features of psoriasis, such as epidermal hyperproliferation, capillary dilatation and large numbers of epidermal CD8+ and dermal CD4+ cells. While in these mice KCs hyperproliferation was observed in the absence of infiltrating leukocytes, epidermal hyperplasia was never seen without skin inflammation. Based on this the authors speculated that suprabasal expression of β_1 integrins in psoriasis may be the cause of psoriasisform changes. Among different integrins shown to be abnormally expressed in hyperproliferative epidermis, a more detailed analysis of the $\alpha_2\beta_1$ integrin will be presented in this section.

$\alpha_2\beta_1$ (CD49b/CD29) integrin is the major collagen I receptor and is expressed on endothelial cells (ECs), KCs, and T cells. This integrin has been shown to participate in different cell functions as cell migration and reorganization, vascular morphogenesis and in cell survival (Senger, Claffey et al. 1997). On ECs, $\alpha_2\beta_1$ is the principal receptor for interstitial collagen I. Senger et al demonstrated that vascular endothelial growth factor (VEGF) potently induced $\alpha_2\beta_1$ expression on dermal microvascular ECs (DMECs) and that this integrin promoted neovascularisation and capillary lumen formation *in vitro* (Senger, Claffey et al. 1997). $\alpha_2\beta_1$, on the other hand, markedly inhibits VEGF-driven angiogenesis *in vivo,* and has been shown to suppress leukocyte infiltration and edema in several mouse models of inflammation (de Fougerolles, Sprague et al. 2000; Senger, Perruzzi et al. 2002).

In the intact epidermis, $\alpha_2\beta_1$ integrin is mostly confined to basal KCs and mediates cell-cell adhesion, possibly by interacting with other integrins, such as $\alpha_3\beta_1$ on adjacent cells (Symington, Takada et al. 1993). However, the exact function of this integrin in healthy skin has not been fully elucidated so far. $\alpha_2\beta_1$ preferentially binds to collagen I and in intact skin these two components are separated by the basal membrane, which is predominantly composed of collagen IV and laminin. $\alpha_2\beta_1$ can also bind to these two proteins, but these interactions are much weaker (Parks 2007). Moreover, α_2 knock-out mice do not present particular skin defects, thereby making the role of this integrin even more mysterious in intact skin. A possible

explanation for the presence of this integrin in healthy skin may derive from its important role in wound healing. It has been shown that after an epidermal lesion $\alpha_2\beta_1$ is required for KCs adhesion on dermal collagen I and activation of other factors necessary for wound closure. Therefore, in intact skin, $\alpha_2\beta_1$ may be a standby condition waiting to respond to injury. In situations where the epidermis is hyperproliferative, such as during wound healing or psoriasis (Hertle, Kubler et al. 1992), aberrant expression of epidermal integrins on suprabasal KCs has been observed. Suprabasal $\alpha_2\beta_1$ integrin expression may thus represent the response of KCs to an inflammatory state (characteristic of wounds and psoriatic lesions) through which KCs are exposed to several pro-inflammatory cytokines. However, when single pro-inflammatory cytokines were added to KCs cultures, there was still no suprabasal integrin expression (Hertle, Jones et al. 1995). The aberrant expression of $\alpha_2\beta_1$ in wounds or psoriatic lesions may likely arise from a complex cascade of events including cytokines and multiple cell-cell interactions.

Regarding T lymphocytes, *in vitro* $\alpha_2\beta_1$ integrin expression has only been observed after long-term activation, and therefore, as for $\alpha_1\beta_1$, this integrin has also been called very late antigen (VLA) -2. However, virus infected T cells after an initial $\alpha_2\beta_1$ expression become rapidly negative for VLA-2 suggesting that this integrin is down regulated after antigen elimination. (Andreasen, Thomsen et al. 2003).

Most of the studies regarding this integrin have been performed on mice. Given the functional importance of integrins in the human setting, a more detailed analysis of $\alpha_2\beta_1$ receptor on human KCs and ECs, and in its role in psoriasis has been investigated as part of this thesis.

Initially, $\alpha_2\beta_1$ integrin expression was analysed on psoriatic sections confirming its expression on dermal ECs and on basal as well as suprabasal KCs. Subsequently, the effects of $\alpha_2\beta_1$ inhibition on keratinocyte hyperproliferation and angiogenesis were studied *in vitro*. Finally, *in vivo* experiments using the AGR xenotransplantation psoriasis mouse model showed that blockade of $\alpha_2\beta_1$ significantly inhibits the development of psoriasis.

These results demonstrated the effect of blocking $\alpha_2\beta_1$ function in psoriasis and suggest a T-cell independent mechanism for inhibiting the development of psoriatic lesions.

4.3.1. Results

4.3.1.1. $\alpha_2\beta_1$ expression on keratinocytes but not on T cells in psoriasis

Aberrant expression of $\alpha_2\beta_1$ has been observed in conditions characterised by epidermal hyperproliferation including wound healing and psoriasis. Immunofluorescence staining of lesional psoriatic sections with an anti-α_2 mAb (green) confirmed that $\alpha_2\beta_1$ integrin was distributed on all sides of basal KCs and also around suprabasal epidermal cells (Figure 4.10A and B). This pattern was clearly different from that of healthy skin and uninvolved psoriatic skin where $\alpha_2\beta_1$ was delimitated to basal cells (Figure 4.10C and D). Long term activated T cells as well activated T cells in chronic inflammatory settings are known to express the $\alpha_2\beta_1$ integrin (Andreasen, Thomsen et al. 2003). Thus, the expression of $\alpha_2\beta_1$ on T cells resident in psoriatic lesions was investigated. Immunofluorescence double staining with mAb to α_2 (green) and CD3 (red) revealed plenty of CD3+ T cells in both epidermal and dermal compartment of established psoriatic lesions, but none of them was expressing the VLA-2 integrin (Figure 4.10A and B). Analyses of symptomless psoriatic skin sections revealed only few T cells in the dermal compartment and none of them was $\alpha_2\beta_1$+ (Figure 4.10D). To confirm the absence of $\alpha_2\beta_1$ expressing T cells in psoriasis, cell suspensions, freshly isolated from lesional and non-lesional psoriatic skin biopsies, were stained with mAb directed to CD3 and $\alpha_2\beta_1$ (Figure 4.10E). Flow cytometry analyses demonstrated no $\alpha_2\beta_1$+/CD3+ cells in none of the two biopsies analysed. Based on these results, the next step was to investigate whether $\alpha_2\beta_1$ was present on peripheral blood cells. Flow cytometry analyses performed on cells isolated from the blood of psoriatic patients and healthy volunteers revealed that peripheral T lymphocytes expressed very low levels of $\alpha_2\beta_1$ integrin and that the two groups had almost the same percentage of VLA-2+/CD3+ cells (Figure 4.10F and G). The CD3-/$\alpha_2\beta_1$+ expressing cells found in these experiments most likely represented natural killer cells, which are known to express the CD49b chain (Hemler 1990; Arase, Saito et al. 2001).

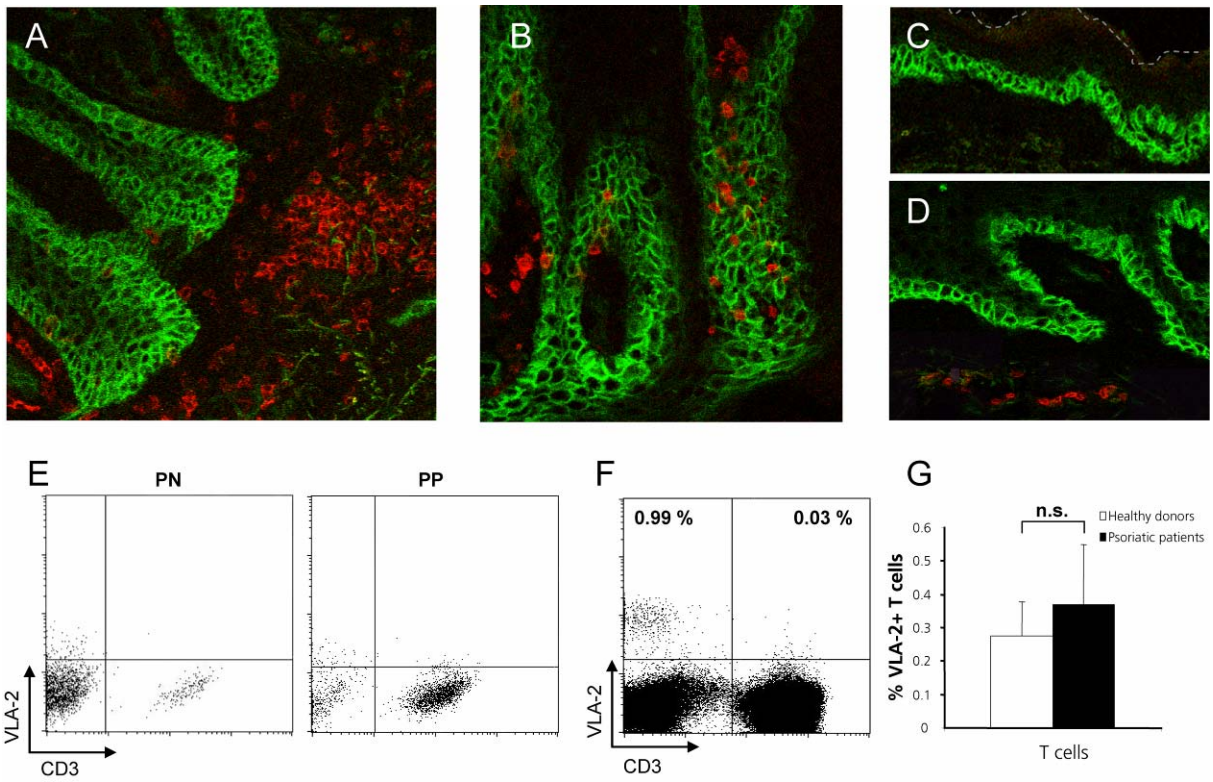

Figure 4.10 VLA-2 expression in lesional psoriatic skin. Two colour immunofluorescence staining using FITC-conjugated anti-CD49b (α_2) mAb (green) and PE-conjugated anti-CD3 mAb (red) on lesional psoriatic skin (A and B), skin of healthy donor (C) and uninvolved psoriatic skin (D). None of the CD3+ T cells (red) present in the psoriatic lesion (A and B) and in the pre-psoriatic skin (D) stained in yellow which would be the consequence of co-localization of CD3 and $\alpha_2\beta_1$ integrin. (E) Flow cytometry experiment with cells freshly isolated from uninvolved (PN) and involved (PP) psoriatic skin biopsies and stained with anti-CD3 and anti-CD49b mAb. (F) Representative example of a flow cytometry analysis performed on peripheral blood T cells of healthy donors (n=7) and psoriatic patients (n=8) and stained with anti-CD3 and anti-CD49b mAb. (G) Peripheral T lymphocytes in the blood of psoriatic patients and healthy volunteers expressed almost the same percentage of $\alpha_2\beta_1$+ cells. Data depicted in G represent mean values plus standard error of the mean (SEM). n.s. = not significant

4.3.1.2. Blocking $\alpha_2\beta_1$ inhibits KCs proliferation on collagen I matrices.

Suprabasal integrin expression may not be a direct consequence of inflammation since in humans it is not induced by intradermal injection of pro-inflammatory (type 1) cytokines (Hertle, Jones et al. 1995). Suprabasal expression may rather correlate with KCs hyperproliferation or with alterations in cell-cell and cell-matrix adhesions. In this context, it has been shown that psoriatic lesions are associated with a general loss of basement membrane integrity (Fleischmajer, Kuroda et al. 2000). In particular, the basement membrane showed marked folding and large gaps or areas with reduced staining for collagen IV and laminins (Fleischmajer, Kuroda et al. 2000). Based on these considerations, it is conceivable that fragmented basement membrane allows contact of basal KCs with dermal collagen I and that this

interaction causes uncontrolled KCs proliferation. To test this hypothesis a proliferation assay with KCs cultivated on collagen I coated wells was performed. Firstly, human epidermal KCs, were isolated from foreskin of healthy individuals and cultivated in serum-free media in the presence of keratinocytes growth factors. Flow cytometric analyses revealed that cultivated KCs expressed high levels (80-90%) of $\alpha_2\beta_1$ integrin, as shown in Figure 4.11A. After 2-3 weeks cultivation, KCs were plated on collagen I coated wells and let adhere over night. 24 hours later anti-α_2 or isotype control mAb were added to the coulters. Proliferation rates were measured by counting the cells (Figure 4.11C) or using the MTT colorimetric assay (Figure 4.11B) on the day of treatment (day 0) and 1, 3 and 5 days after mAb were added. KCs cultivated on collagen I and treated with isotype control mAb proliferated much more if compared to KCs plated on BSA coated wells (control). Addition of anti-α_2 mAb significantly reduced KCs proliferation and induced a cell growth similar to cells cultivated on BSA. These results suggest that the loss of the basal membrane integrity could expose epidermal cell to collagen I and that this interaction induces KCs hyperproliferation in a VLA-2 dependent manner.

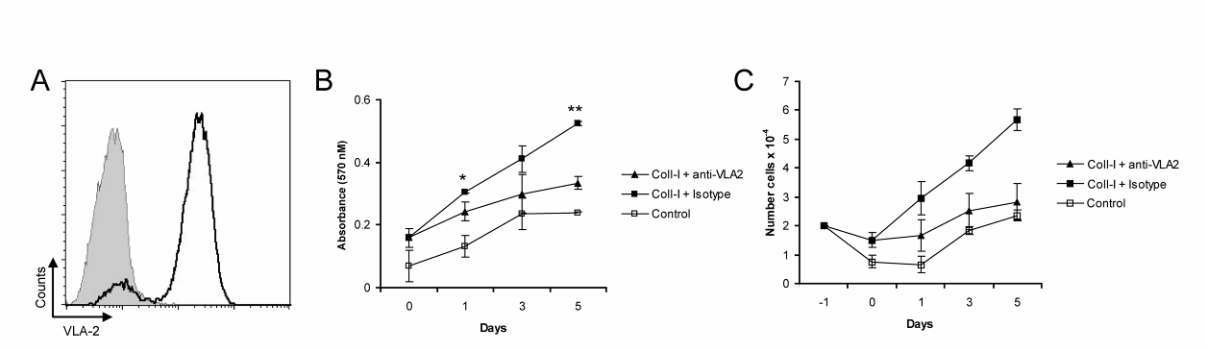

Figure 4.11 Collagen I induces KCs hyperproliferation in a $\alpha_2\beta_1$ integrin dependent manner. (A) Representative (n=3) flow cytometry analysis of cultured KCs. 80-90% of KCs expressed VLA-2 (solid black line). The grey shaded area represents isotype-matched control staining of the same cells. (B) Representative (n=3) MTT proliferation assay performed with KCs cultivated either on collagen I (Coll-I) or on BSA (Control). Anti-VLA2 or isotype control mAb was added 24h after plaiting. (C) Cell counts of cells plated on collagen I or BSA. Anti-VLA2 or isotype control mAb was added 24h (day 0) after plaiting (day -1). Absorbance was measured 24h (Day 0) and 1, 3 and 5 days after plaiting.
*, p =0.048; **, p=0.006

4.3.1.3. $\alpha_2\beta_1$ blockade suppresses dermal microvascular ECs functions

One typical feature of psoriasis is an abnormal vascular expansion within the dermis, principally induced by vascular endothelial growth factor (VEGF), which is produced by epidermal keratinocytes (Creamer, Sullivan et al. 2002). Findings reported by

Senger et al demonstrated that $\alpha_2\beta_1$ is expressed on VEGF-triggered dermal microvascular ECs (DMECs), and that this integrin is involved in ECs migration and proliferation, and in the formation of new blood vessels (Senger, Claffey et al. 1997). Based on these observations, the next step was to investigate whether $\alpha_2\beta_1$ integrin was expressed on the surface of psoriatic blood vessels. Immunofluorescence double staining of lesional psoriatic sections demonstrated co-expression of CD31 (red) and $\alpha_2\beta_1$ (green) on dermal ECs (Figure 4.12A), suggesting that the high amounts of VEGF present in psoriatic lesions potently induced DMEC expression of $\alpha_2\beta_1$. Previous reports demonstrated that $\alpha_2\beta_1$ integrin present on VEGF-stimulated DMECs was important for cell attachment on collagen I and that α_2-blockade markedly inhibited VEGF-driven angiogenesis in mice (Senger, Perruzzi et al. 2002). In order to test the effect of $\alpha_2\beta_1$ blockade on human DMECs two functional *in vitro* experiments were performed. DMECs were cultivated in the presence of VEGF (20ng/ml) and thereafter $\alpha_2\beta_1$ expression was measured with flow cytometry. Analyses performed after at least one day of stimulation demonstrated that above 75% of treated cells expressed the VLA-2 receptor (Figure 4.12B). Subsequently, the proliferation rate of VEGF-stimulated DMECs was measured after seeding the cells on collagen I coated wells in the presence of anti-α_2 or isotype control mAbs. Cell proliferation was measured 1, 3 and 5 days after seeding using the MTT colorimetric assay. Blockade of $\alpha_2\beta_1$ integrin inhibited attachment of DMECs on collagen I and thereby significantly reduced ECs proliferation compared to isotype control treated cells. DMECs seeded on BSA coated wells presented even more reduced cell growth (Figure 4.12C). Given the importance of $\alpha_2\beta_1$–collagen I interactions during EC migration and tube formation the effect of α_2-blockade on DMECs grown in a tridimensional collagen gel were investigated. Therefore, VEGF-stimulated DMECs were plated on collagen I gels in the presence of α_2-blocking mAb or isotype control mAb. As shown in Figure 4.12D, after 2 days of incubation with isotype control mAb DMECs nicely organised in tube-like structures, whereas addition of anti-α_2 mAb strongly inhibited cell spreading and tube formation. Thus, these data together with the one presented in the previous paragraph established $\alpha_2\beta_1$ as an important integrin for KCs and DMECs functions and suggested its direct involvement during psoriasis development.

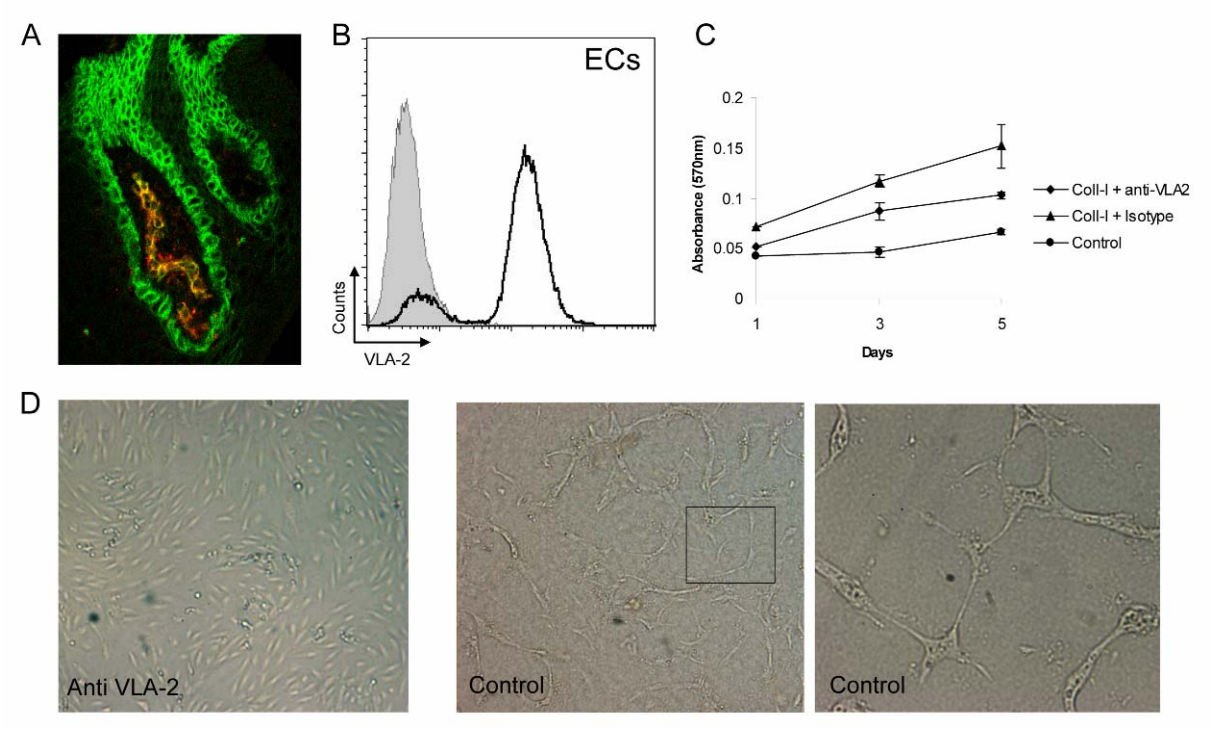

Figure 4.12 $\alpha_2\beta_1$ integrin expression and inhibition on DMECs. (A) Two colour immunofluorescence staining using FITC-conjugated anti-CD49b (α_2) mAb (green) and PE-conjugated anti-CD31 mAb (red) on lesional psoriatic skin. Blood vessel cells appeared in yellow as a consequence of co-localization of CD31 and $\alpha_2\beta_1$ integrin. (B) Flow cytometry analysis of cultured DMECs. 75% of DEMCs expressed VLA-2 (solid black line). The grey shaded area represents isotype-matched control staining of the same cells. (C) Representative (n=3) MTT proliferation assay performed with DMECs cultivated either on collagen I (Coll-I) or on BSA (Control). Anti-$\alpha_2\beta_1$ or isotype control mAb was added one day of seeding. Absorbance was measured 1, 3 and 5 days after plaiting the cells. (D) DMVEc were growth in a tridimensional collagen I gel in the presence of either anti-α_2 or isotype-matched mAb.

By analysing the formation of new blood vessel during psoriasis development in grafts transplanted on AGR mice a final interesting result was obtained. In order to survive human transplants after engraftment onto mice had to be fed by mouse blood. For this purpose both human and mouse vessels had to proliferate at the base of the wound caused during the transplantation and had to fuse in order to form new functional vascular tubes. By staining isotype-control treated grafts excided at day 35 with both anti-human (red) and anti-mouse (green) CD31, the fusion of human and mouse blood vessels could be clearly demonstrated (Figure 4.13).

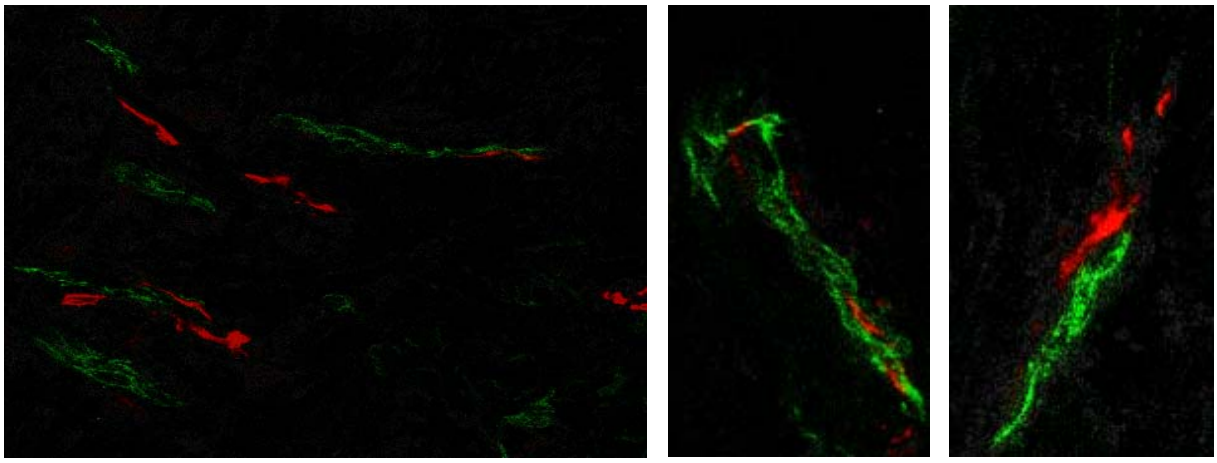

Figure 4.13 Fusion of mice and human blood vessel in the AGR mice model. Immunofulorescence staining using FITC-conjugated anti-human CD31 mAb (green) and PE-conjugated anti-mouse CD31 mAb on isotype-control treated grafts obtained 35 days after transplantation.

4.3.1.4. Antagonism of $\alpha_2\beta_1$ integrin in vivo suppresses psoriasis development

To directly test the importance of collagen I/$\alpha_2\beta_1$ integrin interaction in psoriasis $\alpha_2\beta_1$ function was neutralized during the spontaneous conversion of uninvolved skin into psoriatic skin lesions in AGR xenotransplantation mouse model. Transplanted mice were treated with either neutralizing Ab to the α_2 integrin subunit or isotype-matched control Ab. 5 weeks after transplantation animals were sacrificed and grafts were processed for immunohistochemical analyses. Transplants injected with anti-α_2 mAb presented acanthosis and papillomatosis indices similar to grafts treated with anti-TNF-α, a current standard in anti-psoriatic treatment, and to uninvolved PN skin of the same patient (Figure 4.14), whereas skin grafts from mice receiving isotype-matched control Ab developed into full-fledged psoriatic lesions. Treatment with neutralizing anti-α_2 mAb significantly reduced skin inflammation. Likely, inhibition of epidermal hyperproliferation caused the release of less pro-inflammatory cytokines by KCs and consequently reduced the expansion and the migration into the epidermis of pathogenic T cells. These results indicate the critical role of collagen I/$\alpha_2\beta_1$ integrin interaction for the development of psoriasis and demonstrates that psoriasis can also be blocked using a T cell-independent approach.

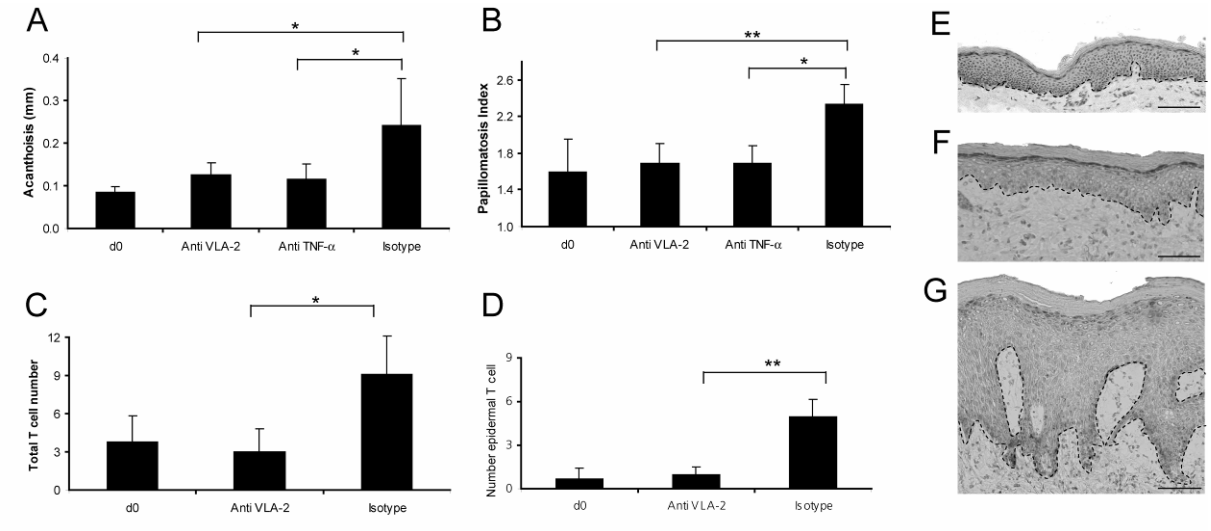

Figure 4.14 Anti-$\alpha_2\beta_1$ treatment inhibits development of psoriasis. (A and B) Acanthosis and papillomatosis indices in skin grafts before transplantation (d0) onto AGR129 mice and after 35 days treatment with either anti-α_2 or isotype-matched control antibody. There was a statistically significant reduction of psoriasis acanthosis and papillomatosis indices in grafts of mice treated with anti-α_2 compared to isotype control mice. The efficacy of the treatment with anti-α_2 in inhibiting psoriasis development was comparable to anti-TNF-α (infliximab), the „gold standard" in psoriasis treatment. (C) Total and (D) epidermal human CD3+ T cell count in PN skin grafts before transplantation (d0) and 35 days after transplantation and treatment with either anti-human anti-α_2, isotype-matched control or anti-TNF-α (infliximab) mAb. There was a statistically significant reduction of both total and epidermal CD3+ T cells in grafts treated with anti-α_2 compared to isotype control treated transplants. Graphs A-D represent mean values of three independent experiments with PN skin from three different patients. P-values were calculated using the unpaired Student's t test *, p= 0,0003 **, p= 0,0008. Scale bar represent 20μm.

4.3.2. Discussion

Collectively the data presented in this section demonstrate that interaction of $\alpha_2\beta_1$ integrin with collagen I causes KCs and ECs hyperproliferation and that this integrin is directly involved in psoriasis development.

$\alpha_2\beta_1$ integrin is the major receptor for collagen I and is expressed on a variety of cells including ECs, KCs, fibroblasts and platelets (de Fougerolles, Sprague et al. 2000). In healthy skin $\alpha_2\beta_1$ is abundantly expressed by basal KCs and may function in cell-cell adhesion possibly interacting with $\alpha_3\beta_1$ (laminin receptor). *In vitro* studies performed in human skin demonstrated that $\alpha_2\beta_1$ was part of the epidermal repair machinery and that anti-α_2 mAb inhibited cell migration on collagen I and across wounded dermis (Parks 2007). Interestingly, two recent *in vivo* studies performed on α_2 integrin-deficient mice instead demonstrated that epidermal wound healing was

not impaired in these animals, suggesting that in mice KCs may not require $\alpha_2\beta_1$ integrin during wound closure (Grenache, Zhang et al. 2007; Zweers, Davidson et al. 2007). Two explanations were given to the different results obtained in human in respect to that seen in mouse. Firstly, the mAb inhibiting integrin function may produce effects different from those due to the lack of integrin expression. Secondly, there is a different distribution of dermal collagen between human and mice, whereas mouse dermis is rich in loose connective tissue, human dermis is a dense fibrillar collagen matrix. Thus, expression of $\alpha_2\beta_1$ integrin in human skin may be more critical compared to the mouse setting. Apart from the role that $\alpha_2\beta_1$ may play in wound healing, the precise function of this integrin in the epidermis is still unclear. In intact skin $\alpha_2\beta_1$ is expressed on basal KCs and may mediate adhesion to collagen IV and laminin of the basement membrane. However, $\alpha_2\beta_1$ preferentially binds collagen I which is the major collagen component in the dermis. In psoriasis, as in wounding, the basement membrane has been shown to be destroyed presenting gaps where collagen IV and laminin are missing (Hertle, Kubler et al. 1992; Fleischmajer, Kuroda et al. 2000). This finding together with the suprabasal integrin expression observed in the two above mentioned skin alterations suggested that dermal collagen I interacting with basal KCs may induce epidermal hyperproliferation in an $\alpha_2\beta_1$ dependent manner. To specifically address the involvement of the $\alpha_2\beta_1$ collagen I receptor during epidermal hyperproliferation, KCs cultured on collagen I were treated with anti-α_2 mAb or isotype control. The significant growth inhibition by anti-α_2 mAb indicated that $\alpha_2\beta_1$ binding to collagen I may contribute to the abnormal KCs growth of psoriasis and wounding.

In addition to be important regulators of epidermal homeostasis integrins play a key role during inflammation. By promoting cell-matrix and cell-cell interactions integrins favour leukocyte migration through the extracellular matrix-rich environment of peripheral tissues. In the skin the most abundant extracellular matrix component is collagen I, accounting for 75% of skin's dry weight (Fitzpatrick. TB 1987). During the development of a psoriatic lesion resident T cells largely proliferate and migrate from the dermal to the epidermal compartment, suggesting that psoriatic T cells may express the $\alpha_2\beta_1$ receptor in order to move through the dense collagen I matrix. Interestingly, neither in lesional nor in pre-lesional psoriatic skin resident T cell were

shown to express the VLA-2 integrin indicating that T lymphocytes do not need collagen I receptors to migrate through the dermal matrix.

Previous studies already demonstrated the importance of $\alpha_2\beta_1$ integrin for angiogenesis (Senger, Perruzzi et al. 2002) and in particular for the formation of the vascular lumen, as required during vessel maturation (Davis and Camarillo 1996). This study demonstrated that $\alpha_2\beta_1$ integrin was highly expressed in the dermal vasculature of psoriasis, perhaps as a consequence of the high levels of VEGF produced by hyperpoliferative KCs (Creamer, Sullivan et al. 2002). In this study, as already demonstrated for KCs, $\alpha_2\beta_1$-collagen I interactions were shown to be the cause of ECs hyperproliferation, as anti-α_2 mAb markedly reduced the proliferation rate of DMECs cultivated on collagen I coated wells. Additionally, α_2–blockade efficiently inhibited DMECs migration and tube formation, thereby confirming the functional role of this integrin during angiogenesis.

The direct involvement of $\alpha_2\beta_1$ integrin during the development of psoriasis was investigated in the AGR xenotransplantation mouse model. Antagonism of $\alpha_2\beta_1$ function with a specific human mAb demonstrated to markedly inhibit psoriasis development, as assessed by reduction of the acanthosis and papillomatosis indices. Anti-α_2 treatment also significantly blocked the proliferation of T cells and their migration in the epidermal compartment compared to grafts treated wit isotype-matched mAb. Moreover, the efficacy of α_2–blockade was comparable to TNF-α treatment, a benchmark in the therapy of psoriasis.

This study demonstrates the critical role of $\alpha_2\beta_1$-collagen I interactions on cell proliferation. It proposes that the loss of the basement membrane integrity exposes epidermal cells to collagen I and that this interaction induces KCs hyperproliferation in a VLA-2 dependent manner. Moreover, the expression of VLA-2 on epithelial and endothelial cells, but not on lymphocytes, shows that α_2–blockade inhibits psoriasis development in a T cell independent manner. The question whether epidermal hyperplasia is a direct consequence of altered signal transduction in KCs or whether it is mediated by the release of pro-inflammatory cytokines by other resident skin cells, such as DCs or T cells is still not completely elucidated. With respect to the results reported in the previous sections, stressing the importance of T cells during psoriasis pathogenesis and demonstrating that T cell expansion precedes the

development of typical psoriatic changes the more reasonable conclusion is that psoriasis is the outcome of an inappropriate immunocyte-based activation event, together with a defect in KCs, whose combination results in the full psoriatic phenotype.

Chapter 5

Concluding remarks and outlook

5. Concluding remarks and outlook

Psoriasis is a chronic inflammatory skin disorder affecting 2-3% of the population worldwide. The most important clinical and histological features of psoriasis are epidermal hyperplasia, increased dilatation and growth of blood vessels together with abnormal T cell proliferation and migration into the epidermis. While psoriasis aetiology remains unknown, considerable understanding of the genetics and immunopathology has been achieved in the last years. T cells together with their secreted cytokines and chemokines are currently believed to be the primary cause of psoriasis. The present study sought to investigate psoriasis immunopathology from different angles by considering the complex cytokine network with a focus on the newly identified IL-23/Th-17 axis and subsequently discussing the importance of the interaction of cells with the skin microenvironment.

Recent discoveries in inflammatory immune diseases are highlighting the role of IL-23, rather then IL-12, as master cytokine potentially playing a role in the persistent inflammatory reaction observed in these disorders. The respective roles of IL-12 and IL-23 in inflammation are likely to be different, as IL-12 stimulates IFN-γ and induces a Th1 lineage shift, whereas IL-23 acts on memory T cells and maintains a recently described population of CD4+ T cells that produce IL-17 (Th-17 cells). Increased IL-23 and IL-17 expression has been identified in the skin of psoriatic patients (Lee, Trepicchio et al. 2004; Steinman 2007) and blocking antibodies to the common p40 subunit of IL-12 and IL-23 have been shown to have clinical benefit in psoriasis patients {Kauffman, 2004 #106; Krueger, 2007 #107}. The present study provides a comprehensive analysis of IL-23 and Th-17 cells and demonstrates their functional relevance in the pathogenesis of psoriasis. IL-23 function was found to be altered in the skin and blood of psoriatic patients and its levels were shown to parallel epidermal hyperplasia implying a functional role for IL-23. Administration of a neutralizing anti-human IL-23 monoclonal antibody inhibited psoriasis suggesting that the beneficial effect of the anti-p40 antibodies, observed when treating psoriatic patients, might be due to the inhibition of IL-23 rather then IL-12. The critical role of IL-23 during the pathogenesis of psoriasis as well other autoimmune diseases makes this cytokine an attractive target for anti-inflammatory strategies. However, it will be

important to determine whether the inhibition of this cytokine will increase the risk of infections.

IL-23 has been shown to play a prominent role in the survival and expansion of Th-17 cells. We saw an enrichment of Th-17 cells in the skin and blood of psoriatic patients compared to healthy donors. IL-17 blockade did not significantly inhibit the development of a psoriatic lesion suggesting that the pathogenic effects of Th-17 cells are not due to IL-17 alone, but potentially due to other cytokines produced by this cell subset. The exact function of IL-17 is to date unclear, but it could include the induction of epithelial and endothelial cells to secrete proinflammatory cytokines and the recruitment of neutrophilis and monocytes into inflammatory lesions (Afzali, Lombardi et al. 2007). Whether any of these mechanisms explains the function of Th-17 cells in psoriasis warrants further investigations. Acosta-Rodriguez et al have recently analysed the Th-17 and the Th1 subsets based on the selective expression of chemokine receptors (Acosta-Rodriguez, Rivino et al. 2007). They found that CCR6 and CCR4 expression identified a population of human memory T cells producing IL-17 but not IFN-γ. Instead, CCR6 and CXCR3 defined a population of Th1 cells that are able to secrete IFN-γ but also IL-17. Based on these results, it would be interesting to investigate whether the Th-17 and Th1 cells found in the skin of psoriatic patients are also expressing these markers. CCR6 has been shown to play a role in the recruitment of T cells to lesional psoriatic skin (Homey, Dieu-Nosjean et al. 2000), whereas CCR4 was found on infiltrating lymphocytes, principally within the psoriatic dermis (Rottman, Smith et al. 2001). It is conceivable that recruitment of T cells into the epidermis is controlled by the expression of specific cell receptors and their respective ligands in the inflamed tissue, rather then from proinflammatory cytokines released by an uncontrolled activation of the immune system.

Further studies are required to clarify the initial signal leading to the dominance of either IL-12 or IL-23 production by the innate immune cells and thus define the functional role and the relative contribution of the Th1 and Th-17 subset in the development of psoriasis.

As mentioned earlier, two typical features of psoriasis are epidermal hyperplasia and massive proliferation and infiltration of T cells in the epidermis, but which is the link between activated keratinocytes and immunocytes during psoriasis onset?

STAT-3 seems to be a major mediator. STAT-3 is essential during wound healing, however an uncontrolled activation of STAT-3 can induce the development of tumours or psoriasis (Sano, Chan et al. 2005)(Yu, Kortylewski et al. 2007). In fact, mice with a constitutive active form of STAT-3 in keratinocytes developed skin lesions resembling psoriasis. Interestingly, a recent study demonstrated that IL-22, a cytokine secreted by Th-17 cells, induced epidermal acanthosis through the activation of STAT-3 and that IL-22$^{-/-}$ mice had significantly less activated STAT-3 and a decrease of total epidermal thickness. These findings provide a further support for the important role of the IL-23/Th-17 axis in psoriasis and suggest that epidermal hyperplasia is the result of lymphocyte infiltration and inflammation in this disease.

Besides the effect of various cytokines in the development of psoriasis, adhesive interactions appear to be important by promoting cell attachment and migration through the inflamed tissue. In this context, the inhibition of specific receptor/ligand interactions may represent another target in the treatment of inflammatory disorders. We showed that by blocking the interaction of $\alpha_1\beta_1$ on T cells with collagen IV in the basement membrane the migration of lymphocytes into the epidermis could be inhibited thereby preventing the development of a psoriatic lesion in the AGR xentotranplantation mouse model (Conrad, Boyman et al. 2007). In this study, we also demonstrated the relevance of other integrins and cell-matrix interactions in psoriasis. Specifically, we showed that interaction of $\alpha_2\beta_1$ with dermal collagen I caused keratinocytes and endothelial cells hyperproliferation and that by blocking this interaction the development of psoriasis could markedly be inhibited. With these findings we introduced a potential T-cell independent mechanism for inhibiting the development of psoriatic lesions and demonstrate the importance of cell-matrix interactions as a causative factor in psoriasis pathogenesis. We hypothesize that abnormal integrin expression, observed in the hyperproliferative epidermis, induces a release of proinflammatory cytokines and growth factors contributing to the inflammation during psoriasis onset.

In conclusion, excessive inflammation in psoriasis may be the outcome of different aberrant mechanisms in the host defence or tissue repair involving not only one single cell or cytokine, but a variety of different factors. The elucidation of specific biological pathways as well as other susceptibility genes will provide a solid basis for the development of new targets for the treatment of psoriasis.

Chapter 6

Material and methods

6. Material and methods

6.1.1. Animal experiments

6.1.1.1. Animals and patients

Animal experiments were performed using AGR 129 mice, which are deficient in type I (A) and type II (G) IFN receptors, in addition to being RAG-$2^{-/-}$. AGR 129 mice show immature NK cells with severely impaired cytotoxic activity in vitro and in vivo (unpublished data) due to a deficiency in type I (A) and type II (G) IFN receptors. In addition, they lack all B and T cell functions as a result of homozygous deletion of the Recombinase Activating Gene 2 (Rag2). AGR mice have been previously described as a clinically relevant psoriasis xenotransplantation mouse model (Boyman, Hefti et al. 2004; Nestle, Conrad et al. 2005; Conrad and Nestle 2006) (Conrad, Boyman et al. 2007). Mice were kept pathogen free throughout the study. Animal studies were approved by the Kantonale Veterinaeramt of Zurich and human studies by the Ethical Committee of the Kanton Zurich.

6.1.1.2. Patients and transplantation procedure

Keratomes (6 x 2 x 0.04 cm) of plaque psoriasis or uninvolved prepsoriatic skin (approx. 5 cm from the leading edge) were obtained from the buttocks or upper thighs of patients with plaque-type psoriasis after informed consent was obtained. Patients did not receive topical or systemic therapy for at least 4 weeks before the study. Mice were 7 weeks to 9 months of age. The AGR xenotransplantation mouse model was performed as published (Boyman, Hefti et al. 2004; Nestle, Conrad et al. 2005; Conrad, Boyman et al. 2007). During transplantation animals were kept under inhalation anaesthesia with Sevoflurane (Sevorane, Abbott AG, Switzerland). After plucking the hair from the backs of the mice, human skin samples measuring 0.5 x 0.5 x 0.04 cm were transplanted using an absorbable tissue seal (Vet-Seal, B. Braun Medical AG, Switzerland), (Figure 6.1A). Grafts were covered with an antiseptic non-adhering dressing (Adaptic, Johnson & Johnson Gateway, England) and fixed with a self-adherent tape (Mefix, Mölnlycke Health Care AB, Sweden). Dressings were removed after 7 days. Mice were sacrificed 5 weeks after engraftment, and skin

transplants snap-frozen in liquid nitrogen and stored at -80°C for histological (Figure 6.1B-F) or mRNA expression analysis. For time course experiments, mice were scarified and grafts were removed 7, 21 and 35 days after transplantation.

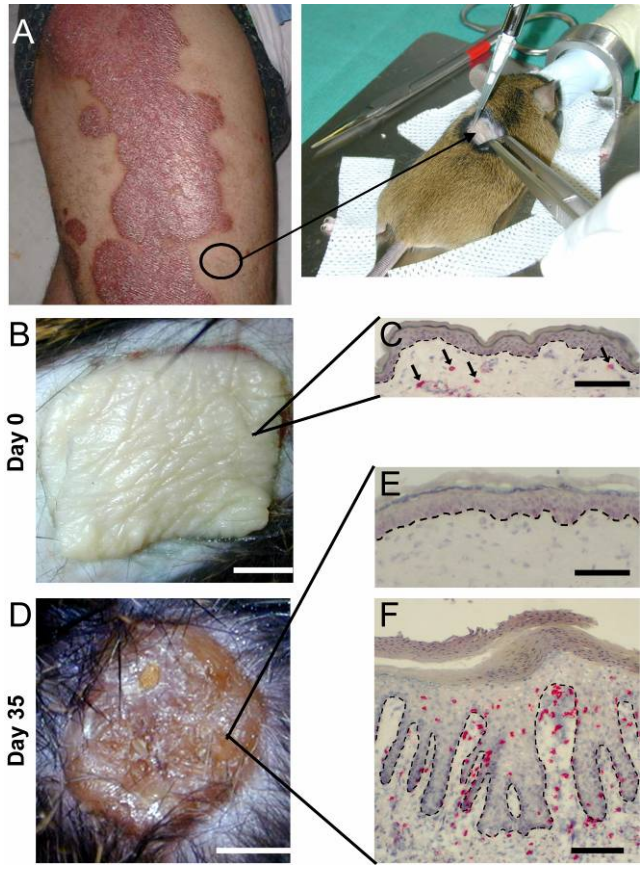

Figure 6.1 AGR xenotransplantation mouse model. (A) Uninvolved psoriatic skin of a patient with psoriasis is transplanted on the back of AGR mice. (B, C) Macroscopic view and hemalaun/CD3 staining of PN skin on the day of transplantation and (D and F) 35 days after engraftment. (E) Hemalaun/CD3 staining of control skin grafts on day 35 after transplantation. Skin of healthy donor fails to convert into psoriasis. In red: CD3+ T cells. Bars B-F: 100 μm

6.1.1.3. Immunohistochemistry staining

Cryopreserved skin specimens were fixed for 10 min in acetone, stained with hemalaun for no longer then 1 min and cleared immediately in sterile water. Immunochemistry was carried out on cryostat sections to detect CD3 positive cells and isotype matched control antibodies were used to asses unspecific binding. Unconjugated mAbs to CD3 or IgG_1 isotype control (both obtained from BD, Bioscience, USA) were used followed by signal amplification using sequential incubations with rabbit anti-mouse IgG xenoantibodies and alkaline phosphatase anti-alkaline phosphatase complexes (APAAP). All the stainings were performed as previously described (Boyman, Hefti et al. 2004; Nestle, Conrad et al. 2005) (Conrad,

Boyman et al. 2007) and performed by the immunohistochemistry laboratory at the Dermatological Clinic of Zurich.

6.1.1.4. Immunofluorescence staining

Cryopreserved skin specimens were fixed for 10 min in acetone and then blocked for 15 min with normal serum (using the serum of the animal where the second Ab has been generated). Tissue sections were incubated with 200 μl of unconjugated mAbs diluted in 0.1% Tween-20/PBS for 1 hour, washed three times in PBS and incubated with 200 μl of conjugated mAbs diluted in 0.1% Tween-20/PBS for an additional hour. For directly labelled mAbs, acetone fixed tissue sections were directly incubated with 200 μl of conjugated mAbs diluted in 0.1% Tween-20/PBS for 1 hour and washed three times in PBS. Finally, sections were mounted onto cover slides with Moviol (Calbiochem. California) and analysed under a confocal laser scanning microscope (Leica, Germany).

6.1.1.5. Histological assessment and quantification experiments

Histological quantification experiments represent the mean of three random fields using a 400-fold magnification. Quantification of human CD3+ T cells and $\alpha_1\beta_1$+ mononuclear cells in uninvolved and lesional psoriatic skin as well as normal skin was performed using immunohistochemistry, while CD3+ T cells in transplantation experiments were quantified using immunofluorescence (in the transplant sections CD3 cells could not be stained with the immunohistochemistry APAAP method, because the transplants were treated with mouse anti-human antibodies). Subepidermal T cells were defined as CD3+ T cells less than 20 μm below the basal membrane. Typical histopathological features of psoriasis include signs of papillomatosis (wavy aspect of the epidermis with upwards extension of dermal papillae), acanthosis (thickening of the epidermis), hyperkeratosis (thickening of the stratum corneum) and parakeratosis (presence of nucleated cells in the stratum corneum). These typical psoriatic features of the epidermis were quantified using the papillomatosis and acanthosis indices as previously described (Fraki, Briggaman et al. 1983). Acanthosis is defined as the maximal epidermal thickness measured from the tip of the rate ridges to the border of the epidermis. The papillomatosis index is

defined as the ratio between adjoining maximal and minimal thickness of the epidermis (elongation of rete ridges, representing ratio of dermo-epidermal border to skin surface). The indicated values of both indices were determined using an ocular micrometer and represent the mean of ten random areas of each sample. Criteria for PN skin with active immune compartment includes a papillomatosis index > 2.0 and the presence of intraepidermal T cells as evaluated in histological sections taken from PN skin.

6.1.1.6. Neutralization studies

Dosage and route of administration of the reagents applied was deduced from therapeutic trials in humans and based on previous data with anti-human mAbs (Boyman, Hefti et al. 2004; Nestle, Conrad et al. 2005; Conrad, Boyman et al. 2007).

Table 6.1 Treatment applied in the animal experiments

mAb	**Isotype**	**Dose (μg)**	**Volume/Route**	**Time Frame**
Anti- IL-23	mIgG1[a]	1000	0.5 ml/s.c	Biweekly
Isotype anti-IL-23	mIgG1	1000	0.5 ml/s.c	Biweekly
Anti-IL17	rIgG1[b]	1000	0.5 ml/s.c	Biweekly
Isotype anti-IL-17	rIgG1	1000	0.5 ml/s.c	Biweekly
Anti-TNFα	Humanised mIgG1	1000	0.1 ml/i.v.	Biweekly
Anti-α_1	mIgG1	1000	0.16 ml/s.c	Twice weekly
Anti-α_2	mIgG1	1000	0.16 ml/s.c	Twice weekly
Isotype anti-α_1/α_2	mIgG1	1000	0.16 ml/s.c	Twice weekly

[a] mIgG1, murine IgG1. [b] rIgG1, rat IgG1

Antipsoriatic treatments versus controls were carried out on parallel grafts from the same subject and statistically analysed in paired fashion.

Anti-IL-23, anti-IL-17 and isotype matched mAbs were kindly provided by Schering-Plough Biopharma, Paolo Alto, CA. Anti-TNFα (Infliximab) was purchased by Centocor, Malvern, Pennsylvania and anti-α_1/α_2 and isotype matched mAb by Biogen Idec, USA.

6.1.2. Cells and cell culture

6.1.2.1. PBMC isolation

Buffy coats from healthy donors or whole blood from psoriatic patients were diluted 1:1 in PBS. Peripheral blood mononuclear cells (PBMCs) were isolated by placing 10 ml Ficoll-Paque (Amersham Pharmacia Biotech, Sweden) at the bottom of a tube and layering 40 ml diluted blood above. The density gradient was generated following centrifugation at 1800g, for 20 min at 20°C. The lymphocyte layer was removed and washed three times with PBS at 453g, for 5 min to remove platelets. Cells were used either for direct flow cytometry staining or for further applications.

6.1.2.2. Sorting of CD3+ cells

Freshly isolated PBMCs or $\alpha_1\beta_1$+ cells were isolated by depletion of non-T cells using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Germany). Cells were first resuspended for 10 min at 4°C in 40 μl of MACS buffer (0.5% BSA, 2mM EDTA in PBS) containing 10 μl Biotin-Antibody Cocktail (anti- CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A) per 10^7 cells. Afterwards, labelled cells were incubated for an additional 15 min at 4°C in 30 μl of MACS buffer containing 20 μl anti-biotin Microbeads. After washing, cells were diluted at a concentration of 10^8 cells per 0.5 ml of MACS buffer and sorted with AutoMACS. The negative fraction was collected. Purity of each T cell population was over 90%, as assessed by flow cytometry analysis after staining aliquots of the cell fractions with a FITC conjugated mAb to CD3 (BD, Bioscience, USA).

6.1.2.3. Isolation of dermal and epidermal cells from psoriatic skin samples

Epidermal and dermal cells were isolated from skin keratome biopsies of plaque psoriatic lesions, uninvolved psoriatic skin or normal skin specimen. Small skin pieces (5 x 5 mm) were first incubated in dispase II (Roche Diagnostics, Switzerland) at 4°C overnight. Dermal sheets were then carefully separated from epidermal sheets, cut into small pieces (1 x 1 mm), incubated with an enzyme cocktail consisting of collagenase, hyaluronidase (Sigma-Chemie, Switzerland), and DNAse

(Boehringer-Mannheim, Germany) for 1 h at 37°C on a shaker in order to obtain a cell suspension. Epidermal T cells were obtained from epidermal sheets by treatment with trypsin 0,05%/EDTA 0,02% for 30 min at 37°C. Cells were stained for cytokines and surface markers as described in the *Flow cytometry analysis* section.

6.1.2.4. Isolation of keratinocytes from foreskin

Foreskins of newborns were washed in PBS, cut in small (0.5 x 0.5 cm) pieces and incubated in dispase II (Roche Diagnostics, Switzerland) at 4°C overnight. Epidermal sheets, after separation from the dermis, were digested in trypsin 0,05%/EDTA 0,02% (Biochrom AG, Germany) for 30 min at 37°C. Cells were washed and resuspended in serum-free keratinocyte (KC) medium containing low calcium concentration and supplemented with human epidermal growth factor (hEGF)(Keratinocyte-SFM, Gibco, Switzerland). Cells were incubated at 37° C in a humidified atmosphere with 5% CO_2. After one week, medium was removed and replaced with fresh medium. Afterwards, medium was changed every 3 days.

6.1.2.5. Dermal microvascular endothelial cells (DMECs) culture

DMECs were purchased by Cambrex and cultivated in EGM-2-MV medium containing the following growth supplements: BBE (bovine brain extract), hEGF, hydrocortisone, 5% FBS (fetal bovine serum), VEGF (vascular endothelial growth factor), IGF-1 (insulin-like growth factor 1) and ascorbic acid (all purchased from Cambrex, USA).

6.1.3. Cell activation and cell analysis

6.1.3.1. Generation of VLA-1+ cells

After isolation, PBMCs were cultured in RPMI 1640 medium (Gibco, Switzerland) containing L-glutamine (Invitrogen, Switzerland) supplemented with 8% heat-inactivated human AB-serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Pen-Strep, Invitrogen, Switzerland). After plating, cells were stimulated with 2 µg/ml phytohemagglutinin (PHA) and 50U/ml recombinant human IL-2 (R&D Systems, UK). Three days after the initial stimulation, new medium and 50 U/ml recombinant human

IL-2 was added, and subsequently replaced twice a week. Cultured cells were analyzed weekly for co-expression of CD3 and using flow cytometry. As soon as $\alpha_1\beta_1$ expression on cultured T cells exceeded 60% (after approx. 4-6 weeks), T cells were isolated by depletion of non-T cells using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Germany).

6.1.3.2. T cell activation with rhIL-23

Purified T cells were activated by using the T cell activation/Expansion kit (Miltenyi Biotec, Germany). T cells were incubated with anti-Biotin MACSiBead particles containing biotinylated antibodies against human CD2, CD3 and CD28 (bead-to-cell ratio 1:2) and cultured in Iscove's modified medium (Invitrogen, Switzerland) with 20% heat inactivated FCS (Sigma, Switzerland), 1% L-glutamine, 1% penicillin and streptomycin and 50mM 2-ME. Human recombinant IL-23 (rhIL-23) (generausly provided by Schering-Plough Biopharma, Paolo Alto, CA) was added at the beginning of the cell culture at a final concentration of 100 ng/ml. Cells were harvested after 1, 2 and 3 days and stained for cytokines and surface markers as described in the *Flow cytometry analysis* section.

6.1.3.3. Flow cytometry analysis

For flow cytometry staining, cells were resuspended in 50 µl of FACS buffer (2% FCS, 0.5 mM EDTA in PBS) containing 1 µl (if not otherwise mentioned) of each surface mAb and incubated for 30 min at 4°C. Afterwards, cells were washed twice (2 min 453g) with FACS buffer and fixed with FACS fix solution (2% formaldehyde solution 36% in FACS buffer). For intracellular staining of cytokines, cells were incubated with PMA (phorbol-12-myristate-13-acetate, 50 µg/ml) and ionomycin (0.5 µg/ml) for 5 hours, adding brefeldin A (5µg/ml) after 1 hour. Cells were first surface stained and fixed (as described above), and then suspended in 50 µl permeabilization buffer (0,1% saponin in PBS) containing mAbs against intracellular cytokines and incubated for 30 min at 4°C. Afterwards cells were washed twice (2 min 453g) with permeabilization buffer, once with FACS buffer and fixed with FACS fix solution. Cells were then analyzed using a FACSCalibur flow cytometer and data

were processed using CellQuestPro (both Becton Dickinson). mAbs used for flow cytometry are listed in appendix I.

6.1.4. In vitro assays

6.1.4.1. Migration assay

Migration assays were performed as follows: cell culture inserts with 5 µm pores (Transwells, Corning, Netherlands) were coated with 25µg/ml collagen IV (Sigma, USA) overnight at 4°C, washed twice with PBS, and blocked with 1% BSA (bovine serum albumin) for 30 minutes at 37°C. 600 µl of RPMI 1640 medium without AB-serum was added to the lower compartment supplemented with SDF-1α (R&D Systems, UK) at indicated concentrations. Cells were adjusted to $2x10^6$/ml in RPMI medium without AB-serum and 100 µl of cell suspension supplemented with 25 µg/ml murine anti-human anti-α1 or isotype control mAb was added to the inserts, respectively. After 1 or 4 hours migrated cells were collected and processed for flow cytometry analysis.

6.1.4.2. Apoptosis assay

The Annexin V/ PI assay takes advantage of the fact that phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis and that the annexin V protein has a strong, specific affinity for PS. Therefore Annexin V is used to quantitatively determine the percentage of cells undergoing apoptosis, while propidium iodide (PI) is excluded by viable cells with intact membranes and thereby distinguishes apoptotic cells with intact membranes from lysed, necrotic cells. For this assay the Annexin V FITC Apoptosis Detection Kit I (BD, Bioscience, USA) was used. Cells were washed twice with cold PBS and then resuspended in 1x binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $Cacl_2$) at a concentration of 1x 10^6 cells/ml. 1x 10^5 cells were stained with 5µl of Annexin V-FITC, 10 µl PI (50 µg/ml PI in 1x PBS) and 1 µl CD3 APC and incubated for 15 min at room temperature in the dark. Afterwards, 400 µl of 1x binding buffer were added to each tube and analysed within 1 hour with flow cytometry.

6.1.4.3. Proliferation assay

24-well cell culture dishes (Falcon, USA) were coated with 25µg/ml rat-tail collagen I (BD Bioscience, USA) in sterile 10 mM acid acetic overnight at 4°C. Wells were washed twice with PBS and blocked with 1% BSA for 30 minutes at 37°C. Control wells were coated with 1% BSA for 30 minutes at 37°C. $2x10^4$ cells/well were seeded on coated wells and 1 day later 25 µg/ml murine anti-human anti-α_2 or isotype control mAb were added. In order to measure cellular proliferation a MTT assay was performed. The MTT proliferation assay is a colorimetric test for the measurement of cell metabolism and approximately of cell proliferation based upon the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). 1, 2, 4 and 6 days after plating, medium was replaced by 250 µl of new medium and 20 µl of MTT solution (5mg/ml; Sigma, USA) was added to each well and incubated for 1 hour at 37°C. At the end of the incubation period the medium was removed and the reduced salt was solubilised by adding 200 µl SDS solution (10% SDS) and 200 µl acid/alcohol solution (5% formic acid in isopropanol) to each well and incubating for 5 min at 37°C. Absorbance was measured on an ELISA reader at a wavelength of 570 nm with a background subtraction at 630 nm.

6.1.5. Angiogenesis assay

This assay was adapted from that of Montesano et al. (Montesano, Orci et al. 1983). 48-well cell culture dishes (Falcon, USA) were coated with 25 µg/ml rat-tail collagen I (BD Bioscience, USA) in sterile 10 mM acid acetic overnight at 4°C. Wells were washed twice with PBS and blocked with 1% BSA for 30 minutes at 37°C. Control wells were coated with 1% BSA for 30 minutes at 37°C. $2x10^4$ DMECs/well were seeded on coated wells. When cells were 80% confluent, wells were rinsed with PBS and given 60 µl of a collagen gel. Gels were prepared by mixing seven parts of 1.4 mg/ml collagen I stock in sterile 10 mM acid acetic with one part 10x Eagle Medium (Gibco, Switzerland) and two parts 11.8 mg/ml sodium bicarbonate stock on ice. Cultures were incubated until gels had solidified (10-15 min) and then given 440µl of EGM-2MV medium with 20 ng/ml VEGF (Peprotech, USA).

6.1.6. Genomics

6.1.6.1. RNA isolation

Skin specimens were homogenised with an electrical homogeniser using a total of 1 ml of Trizol extraction buffer (Invitrogen, California). Subsequentially, RNA was extracted according to the manufacturer's protocol (Invitrogen, California, Cat. No. 15596-026). The RNA was then reverse transcribed into single-strand cDNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche, Switerland, Cat. No. 11 483 188 011) following the protocol of the manufacture.

6.1.6.2. Primer sequence for real time PCR

In order to obtain human specific primers, the human and mouse mRNA sequence for the same gene were first aligned online using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Afterwards, primers were designed on the selected human specific mRNA region using the Primer3 online program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were ordered by Microsynth (Balgach, Switzerland) and diluted in H_20 to a final concentration of 20 pmol/μl. IFN-γ and TNF-α were purchased by Search LC, GmbH Heidelberg.

Table 6.2 Primers used for IL-23p19 detection

TARGET	**PRIMER**	**SEQUENCE (5'- 3')**
Human GAPDH (hGAPDH)	Forward Primer	ATTGCCCTCAACGACCACTTTG
	Reverse Primer	TTGATGGTACATGACAAGGTGCGG
Human IL-23p19 (hIL-23p19)	Forward Primer	CAGCAACCCTGAGTCCCTAA
	Reverse Primer	TCAACATATGCAGGTCCCACT
Human IL-17 (hIL-17)	Forward Primer	ATGGTCAACCTGAACATCCA
	Reverse Primer	TCTCTCAGGGTCCTCATTGC
Human IFN-γ (hIFN-γ)	Primers	Unknown (purchased by Search LC)
TNF-α (hTNF-α)	Primers	Unknown (purchased by Search LC)

6.1.7. Quantitative real-time (RT)-PCR

For the mRNA quantification by RT-PCR, the following reaction-mix components were used:

Table 6.3 RT- PCR component mix

	VOLUME/ CAPILLARY µl	
Reagents/[Mg^{2+}]	**2mM**	**3mM**
H_2O	7.1	6.7
Mg^{2+}	0.4	0.8
Primer forward	0.25	0.25
Primer reverse	0.25	0.25
DNA Master SYBER green	1	1
cDNA	1	1
Total Volume	10	10

LightCycler FastStart DNA Master Syber Green I was purchased from Roche Diagnostic. Mg^{2+} concentration was optimised for each target. RT-PCR was performed on the Light Cycler 2.0 (Roche Diagnostic, Switzerland).

The detecting run was constructed following the manufacturer's experimental protocol, optimizing the amplification settings for each target.

Table 6.4 RT-PCR amplification protocol

PRIMER	**[Mg^{2+}]**	**ANNEALING TEMPERATURE (C°)**	**ELONGATION TIME (Seconds)**	**NUMBER OF CYCLES**
hGAPDH	2mM	62	15	40
hIL-23p19	2mM	57	8	48
hIL-17	3mM	57	5	60
hIFN-γ	2mM	68	16	55
hTNF-α	3mM	68	16	55

At the end of each run, all the data were analysed using the Standard Curve Method. mRNA concentration was determined in relation to an external standard curve prepared by amplification of 10-fold dilutions of a given concentration of the target gene. Each mRNA concentration was further normalised with the hGAPDH concentration of each sample.

Chapter 7

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Monocolonal antibodies used for FACS staining

ANTIGEN	LABEL	DILUTION	COMPANY
CD3	FITC, Cy3, APC	1:50	BD Bioscience
CD4	PE, Cy3, APC	1:50	BD Bioscience
CD8	FITC, APC	1:50	BD Bioscience
CD11c	APC	1:50	BD Bioscience
HLA-DR	PE	1:50	BD Bioscience
CD45RA	Cy3	1:50	BD Bioscience
CD45RO	APC	1:50	BD Bioscience
CD49a	PE	1:10	BD Bioscience
CD49b	FITC	1:10	BD Bioscience
CD56	FITC	1:10	BD Bioscience
Annexin V	FITC	1:10	BD Bioscience
CCR7	FITC	1:50	R&D System
IL-23R	Biotilayted	1:20	R&D System
Streptavidin	PerCP	1:200	BD Bioscience
Cytokeratin	FITC	1:500	Affinty BioReagents
CCR4	APC	1:500	BD Bioscience
NKG2D	APC	1:10	BioLegend
Vδ1	FITC	1:10	Endogen
Vγ9	Cy3	1:200	Beckman Coulter
CD1b	APC	1:10	Miltenyi Biotec
CD103	FITC	1:10	Immunotech
CD1a	PE	1:10	Dako
IFN-γ	FITC	1:10	BD Bioscience
IL-17	PE	1:8	Schering-Plough
isotype-matched control mAbs			

FITC, Fluoresceinisothiocyanat; PE, Phycoerythrin; Cy3, CyChrome-3; APC, allophycocyanin; PerCP, Peridinin-Chlorophyll-Protein

Curriculum Vitae

Personal

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Education

1988 - 1991	Primary School, Music Conservatory G.Tartini of Trieste, Italy
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2003 - 2007	PhD at the Department of Dermatology University Hospital of Zürich PhD Thesis: “New Immune Effector Pathways In Psoriasis” Supervisor: Prof. Dr. med. FO Nestle Thesis supervisor at MNF: Prof. Dr Peter Sonderegger

Publications

Conrad, C., Boyman, O., **Tonel**, G., Tun-Kyi, A., Laggner, U., de Fourgerolles, V., Gardner, H. and Nestle, FO. A critical role for VLA-1 in accumulation of epidermal T cells and the develpoment of psoriasis. *Nature Medicine;* **13** 836-42 (2007).

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